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VOLUME 37 NOVEMBER 1959 NUMBER 11

COMPARISON BETWEEN SEASONAL AND THERMAL ACCLIMATION IN WHITE RATS

II. SURFACE TEMPERATURE, VASCULARIZATION, AND IN VITRO RESPIRATION OF THE SKIN[1]

O. HÉROUX

With the technical assistance of DONNA WRIGHT

Abstract

The effect of cold temperature on the skin was studied on white rats exposed to two different types of environmental conditions. Two groups of adult rats kept in individual cages were continuously exposed for 3 months to constant cold temperature (18° and 6° C) in the laboratory (indoor rats) while other groups of the same colony kept in groups of 10 were exposed for the same length of time to the fluctuating environmental conditions prevailing outside (outdoor rats).

Indoor rats acclimated to 18° C and 6° C showed the same increase in the number of opened capillaries in the ears over the number observed in controls acclimated to 30° C. "Summer and winter" outdoor rats showed the same number of capillaries as the "18° C or 6° C" indoor rats. Signs of injury healing such as thicker epidermis and larger nuclei were found in the ears of all the "6° C" rats but in none of the "winter" rats. While the skin temperature measured at +6° C was slightly higher (0.4 to 1.0° C) in rats acclimated at 6° C than in those at 30° C, it was lower (1.3 to 2.9° C) in "winter" than in "summer" rats. After 28 days of acclimation, the rate of oxygen uptake of the dorsal skin of the foot was lower in "6° C" than in "30° C" rats but after 84 days it was significantly higher in the cold-acclimated rats. Similarly, after 3 months, the respiratory rate of the dorsal skin of the foot was higher in "winter" rats than in "summer" rats.

Introduction

When white rats kept in individual cages were continuously exposed to a constant cold temperature (+6° C) in the laboratory, they developed a degree of cold resistance similar to that of rats exposed in groups of 10 to the fluctuating environmental conditions prevailing outside during the winter (2). While the metabolic adjustments, such as increased peak metabolism and decreased shivering were also similar in indoor and outdoor cold-exposed rats, the insulative adjustments and thermoneutral metabolic rates were quite different. The pelage insulation increased in the outdoor winter rats and remained unchanged in the indoor cold-exposed rats. The metabolic rate measured at 30° C increased in the "6° C" acclimated rats but not in the winter-exposed animals. Moreover, while all the "indoor" rats developed cold injury, there were no external signs of such lesions in the "outdoor" rats.

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
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The present paper describes additional peripheral adjustments which differ in "indoor and outdoor" rats. These adjustments have been characterized by records of the surface temperatures of the back, tail, and foot, counts of the number of capillaries, measurements of the thickness of the epidermis and the cell nuclei diameters in the ear, and finally measurements of the in vitro respiration of the skin of the foot of both "indoor and outdoor" rats.

Experimental Procedures

Animals and Environmental Conditions

A total of 158 adult male Sprague–Dawley rats, of an average body weight of 200 g, were submitted to two different sets of environmental conditions. Ninety-six rats were moved during the winter into constant temperature rooms (46 at 30° C, 4 at 18° C, and 46 at 6° C) and 62 were exposed to the outdoor fluctuating environmental conditions, from October 23 to January 23 during the winter and from May 15 to August 15 during the summer. The average outdoor temperature as well as the methods for holding and feeding the animals have been reported in a previous paper (2).

Methods

The methods for counting capillaries, measuring the epidermis thickness and cell nuclei diameters have been previously described (4). These three measurements were done on sections of tissue obtained on the same animals.

Skin temperatures were recorded at 6° C, (1) on the back at two different locations along the mid-line, one above the shoulder blades (I) and one halfway between the neck and the tail (II); (2) on the tail at 1 inch from the base (I) and halfway between the base and the tip (II); (3) on both the ventral (V) and dorsal (D) surfaces of the foot. Preliminary experiments had shown that the rats should not be disturbed for measurements of skin-surface temperatures; if they are picked up, their skin temperatures rise by 5 to 7° C at the extremities (foot and tail). Skin temperatures on the back, tail, and dorsal surface of the hind foot were therefore obtained by lightly touching the animals with a fine copper–constantan thermocouple, attached to a wooden probe, while the rats were resting in their cages. After these temperatures had been recorded, the hind leg was lifted with the hand and the thermocouple applied to the ventral surface of the foot. The temperatures were recorded after the animals had been transferred to a cage at 6° C for at least 1½ hours.

Q_{O_2} measurements were obtained on the dorsal skin of the foot of some of the animals on which skin temperatures had been previously recorded. The animals were killed by excess of ether and the skin covering the entire dorsal surface of the hind feet was removed by cutting with scissors and lifting the skin and adherent subdermal tissue from that beneath. Two pieces of skin, one from the left paw and one from the right paw, were taken from each rat and weighed immediately on a torsion balance.

After 28 days of exposure to 30° C or 6° C the in vitro respiration of the skin on the foot was measured on two different subgroups of rats. In the first subgroups (I), there were four rats at each temperature, in each case the eight

pieces of skin were pooled and put in a common flask. In the second subgroups (II), eight rats from each temperature were used and again the 16 pieces of skin were pooled and put in a common flask. After 84 days, the rate of oxygen uptake of the skin of the foot was measured on a third subgroup (III) of 16 rats at each exposure temperature; in both subgroups the 32 skins were equally distributed among eight flasks. The in vitro respiration of the skin of the foot was measured on 24 "outdoor winter" rats and on 22 "outdoor summer" rats, for each group the pieces of skin were distributed in five flasks.

Oxygen consumption was first determined at +18° C by the direct method of Warburg, using 100% oxygen as the gas phase. The oxygen consumptions were calculated from readings taken during the period from 90 to 140 minutes after placing the tissue in the isotonic medium with glucose described by Fuhrman (1). During the next 20 minutes the bath temperature was raised to 27.5° C. A 15-minute equilibration period followed and readings were taken again during 40 minutes. The bath was raised to 37° C and the same sequence was again followed. Rates of oxygen uptake are expressed in microliters of gas consumed per milligram of initial wet weight per hour. Preliminary experiments had shown that similar results were obtained if the tissues were incubated at 37° C first and 18° C at the end.

Results

The number of capillaries filled with blood in cross sections of the ears was essentially the same in rats exposed to 18° C and 6° C. But the number of opened capillaries in cold-acclimated rats was much greater than in 30° C-acclimated rats (Table I). In the "outdoor" animals there was no significant difference in the number of capillaries between "summer and winter" rats.

The histologic examination of the ears of "6° C" rats revealed many signs of healing processes following a cold injury. In addition to the increase in size of the cells, nuclei, and nucleoli, there was an increase in the number of mitoses. All these changes have been previously described (4). Moreover, the arrangement of the new cells was disorderly as commonly observed in repair processes. In Table I are reported two of the changes, the increase in thickness of the epidermis and the increase in diameter of nuclei in the "6° C" rats, which were statistically significant.

None of these changes were evident in the ears of winter rats. Both the nucleus diameter and the epithelium thickness were essentially the same after acclimatization to summer and winter conditions, and the layers of cells were normally arranged in both groups, which strongly suggests that cold injury did not develop in these animals throughout their 3 months' exposure.

A glance at the surface temperatures of the skin (Table I) shows that at the six different locations in the indoor rats the temperature was higher in the "6° C" rats than in the "30° C" rats. The chances of this occurring fortuitously are very slight, so that there is little doubt of a real difference in average skin temperature between the two acclimation groups, although only two of these differences (back and tail I) were statistically significant when tested individually.

TABLE I

Effect of indoor and outdoor environments on number of capillaries, thickness of epidermis, diameter of nuclei, and skin surface temperatures

Measurements		Indoor				Outdoor		
		30° C	18° C	6° C	Diff. 6° C − 30° C	Summer	Winter	Diff. W − S
No. of capillaries/mm²	Ear	17.8 ± 5.6† (6)	81.5 ± 15.9 (4)	100.2 ± 11.9 (6)	+82.4 ± 12**	81.8 ± 12.2 (6)	105 ± 10.5 (6)	+ 23.2 ± 18.9
Epidermis thickness (μ)	Ear	21.7 ± 0.7 (12)		58.4 ± 10.0 (12)	+36.7 ± 10**	32.1 ± 3.2 (6)	27.6 ± 1.2 (6)	− 4.5 ± 3.4
Nucleus diameter (μ)	Ear	6.1 ± 0.1 (12)		9.4 ± 0.4 (12)	+ 3.3 ± 0.4**	7.7 ± 0.5 (6)	6.8 ± 0.3 (6)	− 0.9 ± 0.6
Skin temperature (° C)	Back I	33.4 ± 0.21 (12)		34.4 ± 0.28 (12)	+ 1.0 ± 0.35**	34.15 ± 0.32 (10)	31.17 ± 0.64 (10)	− 2.98 ± 0.7**
(measured at +6° C)	Back II	31.4 ± 0.21 (12)		32.0 ± 0.28 (12)	+ 0.6 ± 0.36	31.05 ± 0.32 (10)	29.40 ± 0.64 (10)	− 1.65 ± 0.7*
	Tail I	10.4 ± 0.21 (12)		11.2 ± 0.28 (12)	+ 0.8 ± 0.35*	12.91 ± 0.32 (10)	11.61 ± 0.64 (10)	− 1.30 ± 0.7
	Tail II	9.4 ± 0.21 (12)		9.5 ± 0.28 (12)	+ 0.1 ± 0.35	11.88 ± 0.32 (10)	10.07 ± 0.64 (10)	− 1.81 ± 0.7*
	Foot D	11.0 ± 0.21 (12)		11.4 ± 0.31 (12)	+ 0.4 ± 0.37	13.08 ± 0.32 (10)	10.83 ± 0.64 (10)	− 2.25 ± 0.7**
	Foot V	10.4 ± 0.21 (12)		11.0 ± 0.31 (12)	+ 0.6 ± 0.37	12.77 ± 0.32 (10)	11.17 ± 0.64 (10)	− 1.60 ± 0.7*

NOTE: Number of animals is in parenthesis.
* = Statistically significant at the 5% level.
** = Statistically significant at the 1% level.
† = Standard error.

In contrast to "indoor" rats, the skin temperatures of the cold outdoor groups (winter) were lower than those of the summer group at all locations, all differences, except tail I, being statistically significant.

In all indoor and outdoor groups, within back and tail regions, subregion I significantly exceeded subregion II.

After 28 days of indoor cold exposure, the oxygen uptake of the skin of the foot was lower in "6° C" rats than in "30° C" rats (Table II). The "in vitro"

TABLE II

In vitro respiration of dorsal foot skin of white rats

Environmental conditions	No. days of exposure	No. rats	No. vessels	Av. wet wt. of tissue per vessel, mg	Bath temperatures		
					37° C	27.5° C	18° C
					O_2, μl/hr/mg (wet wt.)		
Indoor							
30° C (I)	28	4	1	586	0.50		0.069
30° C (II)	28	8	1	1333	0.49		0.139
6° C (I)	28	4	1	412	0.38		0.046
6° C (II)	28	8	1	929	0.42		0.084
30° C (III)	84	16	8	536	0.393±0.008†	0.201±0.006	0.072±0.008
6° C (III)	84	16	8	419	0.447±0.008	0.223±0.006	0.065±0.008
					+0.054±0.0012**	+0.022±0.008*	−0.007±0.011
Outdoor							
Summer	84	24	5	1029	0.424±0.013	0.207±0.005	0.074±0.017
Winter	84	22	5	735	0.506±0.009	0.225±0.006	0.070±0.026
					+0.082±0.032*	+0.018±0.008*	−0.004±0.032

* = Statistically significant at 5% level.
** = Statistically significant at 1% level.
† = Standard error.
(I), (II), (III), = number of subgroup.

respiration for the skin of the "6° C" rats was lower at 37° C as well as at 18° C in both subgroups I and II. After 84 days, however, the oxygen uptake appeared to be greater in the "6° C" than in the "30° C" skins. A statistical analysis of the results obtained in subgroups III indicated that the rate of oxygen uptake of the skin of the rats acclimated to "6° C" was appreciably greater ($p<0.01$) at 37° C and perceptibly greater ($p<0.05$) at 27.5° C. No significant difference was obtained, however, between the two groups when the measurements were made at 18° C.

Similar results were obtained for the "outdoor" rats. After 84 days of exposure, the rate of oxygen uptake of the skin of "winter" rats exceeded that of "summer" ones at 37° C and 27.5° C but at 18° C no significant difference was obtained between the two groups.

Discussion

The "outdoor winter" rats resemble the "cold indoor" animals in the greater vascularization of the ear and the higher rate of oxygen uptake of the dorsal skin of the foot but they differ in skin temperature and also in evidence of cold injury. The absence of cold injury in "outdoor" rats was revealed by direct observation and by lack of histological evidence of healing processes, such as disorderly arrangement and hypertrophy of the cells.

The capillary counts in ears of rats exposed to 30° C, 18° C, and 6° C reveal that after a prolonged exposure to either a cool or a cold environment, all the

available capillaries are opened up. The similarity in the number of capillaries in "18° C" and "6° C" rats suggests that even under the colder temperature conditions no new capillaries were formed at the tip of the ear where these vessels have been counted. A similar phenomenon obtained under the outdoor fluctuating environmental conditions; in the "summer" rats, which were exposed on the average to a temperature of 19° C, the number of opened capillaries was essentially the same as in the "winter" rats and both summer and winter counts were quite similar to the ones observed in the rats acclimated to 18° C or 6° C indoors. This shows that after a prolonged or repeated exposure to a cool environment, whether indoors or outdoors, all the capillaries remain open and new vessels do not develop.

The increase in oxygen uptake of the skin of the foot in vitro above the control level, following an initial reduction during the first 4 weeks of cold exposure, parallels the renewal of mitotic activity in the ear epidermis which followed the almost complete arrest of cellular division during the first 21 to 28 days of exposure (4). Whether similar readjustments took place in the back skin covered by fur remains to be demonstrated. It might be significant, however, that essentially the same mitotic duration was found in the epidermis of the back of both "30° C" and "6° C" rats although the cold-exposed animals maintained the skin on their backs at a temperature 3° C lower than did the "30° C" controls (3).

Since the skin of the foot of the "winter" rats also showed a higher oxygen uptake than similar skin of the "summer" rats, it appears that epidermal metabolic adjustments can develop on the foot whether the animals are continuously exposed to cold, as indoors, or intermittently exposed as outdoors.

The higher skin temperature in the "6° C" rats than in the "30° C" rats when both are exposed to 6° C is consistent with the greater heat loss in the cold-acclimated rats already suggested by their greater oxygen consumption. Similarly, the lower skin temperature observed in "winter" rats as compared with the summer controls is also consistent with the reduced heat loss which their lower oxygen consumption and greater pelage insulation suggested.

During the winter, an outdoor rat is not only exposed to fluctuating cold from day to day and even within a day, but the temperature gradually decreases from an average +10° C in October to −10° C in January. In spite of these irregular exposures to gradually decreasing temperature, the "winter" rats were subjected to conditions, which, on the average, induced cold resistance equal to that found in the "indoor 6° C" acclimated rats. The fluctuating nature of the outdoor cold exposure, and the fact that the rats, by huddling, were not always exposed to cold, might, however, explain the absence of cold injury in the ears of "winter" rats. It is conceivable that under these circumstances the exposure was sufficient to lead to increased cold resistance of the epidermal cells but not severe enough at any given time to produce cold injury.

Further work will be required to determine what factors or combination of factors were responsible for the different adjustments observed in the outdoor rats. Besides the temperature and the huddling factors, light intensity will also have to be considered. This new series of investigations will be a study of the

experimental conditions per se while up till now the problem was to determine whether a different set of environmental conditions could induce in white rats the same degree of cold resistance and the same metabolic adjustments as are observed in isolated rats kept in a cold room, without some of the physiological and anatomical changes usually observed under these constant temperature conditions.

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COMPARISON BETWEEN SEASONAL AND THERMAL ACCLIMATION IN WHITE RATS

III. STUDIES OF THE ADRENAL CORTEX[1]

O. HÉROUX AND E. SCHÖNBAUM

Abstract

The rate of production of corticosteroids in vitro as well as the histological picture of the adrenal glands was studied in white rats exposed to cold for 3 months, either indoors at 6° C in individual cages or outdoors during the winter in groups of 10.

Under the indoor cold conditions, the adrenals hypertrophied within 1 week and their weight then remained constant for the following 11 weeks. The hypertrophy was due to an increase in the number of cells in the zona fasciculata. Relative to adrenal weight, the production of corticosteroids in vitro was less in the 6° C rats than in the 30° C controls. Under the outdoor cold conditions, the adrenal weight as well as the number of fasciculata cells remained normal, but the steroid production "in vitro" was greater than in the "summer controls". Since both "indoor" and "outdoor" cold-exposed rats have been shown previously to develop a similar degree of cold resistance as well as a similar capacity for elevating their metabolism through a non-shivering heat production mechanism, it appears that similar degrees of adaptation to cold can exist with different requirements of adrenocortical hormones.

Introduction

In recent years, possible differences between the adjustments required for survival in a laboratory cold room on the one hand and under the normal seasonal changes on the other have received considerable attention. Observations on body weights, heart rates, metabolic rates, shivering, fur insulation, skin temperature, cold injury, vascularization of the ears as well as some endocrine effects have been reported previously (4, 5, 6). In this paper, the studies concerning the adrenal glands are described in detail. Effects of acclimation on the function of the thyroid glands will be dealt with in another paper (7).

Experimental Procedures

One large group of male Sprague–Dawley rats was raised in an environment of 20° C, in groups of 10 rats per cage. All animals received Master Fox cubes and tap water ad libitum. On reaching an average body weight of 220 g, 10 rats were decapitated and their adrenals were weighed on a Roller Smith balance; all the other rats were distributed at random into three groups and exposed during the summer for 3 months to warm or cold environments. Two groups were kept indoors under constant temperature and light conditions, one at 30° C and the other one at 6° C; artificial lights were on from 8 a.m. to 8 p.m. every day and supplied the animals with an illumination in their cage of around 3.7 foot-candles at 30° C and 7 foot-candles at 6° C. The third group

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was kept outdoors, and exposed to natural environmental conditions. The following winter, another group of rats of the same average body weight and coming from the same colony was again divided into three groups, two of which were kept indoors at 30° C and 6° C, respectively, and one was kept outdoors. The "indoor rats" were kept in individual cages while the "outdoor rats" were kept in groups of 10. In individual cages, the rats outdoors would not have survived the rigorous winter conditions (occasionally the temperature goes down to −30° C).

Altogether, excluding the 10 rats that were sacrificed at the beginning at 20° C, there were six different groups of rats:

(*a*) summer 30° C (indoors),
(*b*) summer 6° C (indoors),
(*c*) summer outdoor,
(*d*) winter 30° C (indoors),
(*e*) winter 6° C (indoors),
(*f*) winter outdoor.

After different periods of exposure, 1, 3, 5, and 12 weeks, some of the "winter 30° C" and "winter 6° C" rats were weighed and decapitated as fast as possible. Both adrenals in each rat were dissected without delay and weighed on a Roller Smith balance.

All the other "summer and winter 30° C and 6° C and summer and winter outdoor rats" were killed in the same way after 12 weeks of exposure. Their adrenals were dissected and weighed. On five "winter 30° C and 6° C rats and winter outdoor rats", chosen at random, the water content of the adrenal glands was determined by weighing one gland before desiccation at 90° C and after desiccation until no further changes of the weight were observed. The other gland was fixed in Bouin's fluid. The adrenals from the other animals were stored in a Petri dish, on moist filter paper, until all had been collected.

Histological Examination

After fixation in Bouin's fluid, the adrenals were dehydrated in alcohol, cleared in toluol, and embedded in paraffin (Fisher Tissuemat, melting point 54–56° C). Sections of 10 μ were cut and stained with haematoxylin and eosin. The total thickness of the gland was estimated by counting the sections, assuming their thickness to be constant. Similarly, the thickness of the medulla was found by multiplying by 10 the number of sections in which that zone could be seen. The largest section was located with the aid of an eyepiece micrometer, and projected on a table through a Bausch and Lomb enlarger (magnification ×45). Each zone in the largest section of each gland was outlined and the areas covered by the four different zones were measured with a planimeter. Taking the adrenal as a prolate spheroid, the volumes of the whole gland and of the medulla were calculated with the formula $4/3\pi abc$, πab being the area measured on the largest section and "c" the radius of the third dimension or the thickness divided by 2.

Cells were counted at four different places located at random in the fasciculata zone in an area delimited by an eyepiece square grid covering a surface of 4489 μ^2 at a magnification of 12.5×100. In the last area, the diameter of the first 10 nuclei appearing under the eyepiece micrometer was measured.

Steroid Production in Vitro

The adrenal glands that had been collected in the Petri dish were cleaned, quartered, and preincubated for 30 minutes, then incubated for 1 hour in a Krebs–Ringer bicarbonate glucose medium (9). Subsequently the medium was extracted with methylene dichloride. Corticosteroids were measured by their absorption at 240 mμ (10).

Results

Anatomical Changes

In both 30° C and 6° C rats, the weight of the adrenal glands was linearly related to body weight ($P < 0.01$). An increment of 1 gram of body weight was associated with a corresponding average increment in adrenal weight of 0.098 mg. When differences in body weight were taken into account, the average adrenal weight of "6° C" rats adjusted to the average body weight (310 g) of both groups of rats appeared to increase during the first week, and to remain constant thereafter until the end of the experiment. The regressed weight (3) of the adrenal glands seemed to return towards the initial weight observed in rats that had been kept at 20° C before exposure to 6° C, but the changes were not statistically significant (Fig. 1). At 30° C, the regressed weight of the adrenal glands did not change during the first 5 weeks but it was significantly lower after 12 weeks.

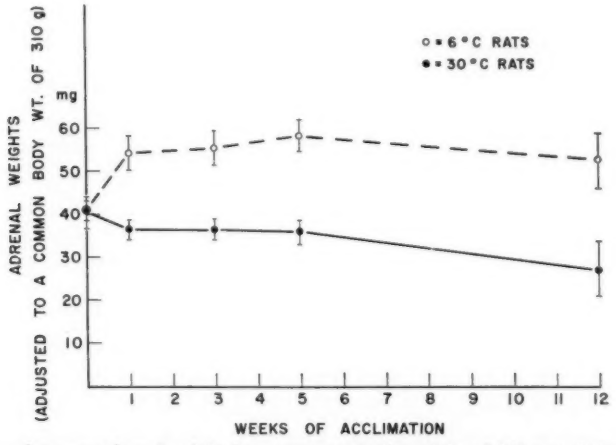

FIG. 1. Average adrenal weight for a common body weight of 310 g at different times during acclimation; ○———○ rats kept at 6° C and ●———● rats kept at 30° C.

The amount of water in the adrenal glands was essentially the same in the three "winter" groups: 65.3% ± 1.2 for the 30° C rats, 66.6% ± 1.2 for the 6° C rats, and finally 67.4% ± 1.2 for the "outdoor rats". There are no statistically significant differences.

The measurement of the different zones in the adrenals of the two "indoor groups" revealed that "6° C rats" had bigger adrenals but that a significant

increase in size was evident only in the zona fasciculata but not in the zona glomerulosa, zona reticularis, or the medulla (Table I). The diameter of the

TABLE I

Areas of adrenal zones

	Adrenal wt., mg	Areas covered by each zone × 45², mm²				
		Total	Glomerula	Fasciculata	Reticularis	Medulla
Indoors						
30° C (5)	20.20±0.90†	12,070±453	1,172±21	4,386±460	4,304±251	2,208±160
6° C (6)	24.78±0.83	14,323±414	1,193±67	6,427±420	4,363±229	2,340±146
Diff. 30° C−6° C	−4.58±1.22**	−2,253±614**	−21±70	−2,041±623**	−59±340	−132±216
Outdoors						
Summer (6)	26.20±2.15	13,670±969	1,237±20	5,350±582	4,560±391	2,523±190
Winter (8)	23.46±1.99	13,588±839	1,184±58	5,588±504	4,416±339	2,275±165
Diff. (S−W)	2.74±2.93	82±1281	53±61	−238±770	144±517	248±251

NOTE: In parenthesis, number of animals.
**Statistically significant at the 1% level.
†Standard error.

nuclei and the number of cells per unit of area in the zona fasciculata were the same in both "30° C and 6° C rats". However, the total number of fasciculata cells in the largest section was larger in the cold-acclimated rats (Table II). The volume of the adrenal medulla expressed as a percentage of the total volume of the adrenal gland was essentially the same in both acclimated groups.

TABLE II

Volume of medulla and number of cells in the fasciculata zone

	Volume of adrenal, mm³	Volume of medulla % of total gland volume	Fasciculata zone		
			Cells/mm²	Total number of cells × 10^{-1}	Diameter of nuclei, μ
Indoors					
30° C (5)	10.09±0.54†	6.82±0.66	8,370±880	3,556±247	6.70±0.10
6° C (6)	13.30±0.49	6.24±0.60	6,980±220	4,459±226	6.92±0.09
Diff. 30° C−6° C	−3.21±0.73**	0.58±0.90	1,390±910	−903±335*	−0.22±0.13
Outdoors					
Summer (6)	12.21±1.16	7.95±0.58	8,507±545	4,547±225	6.41±0.16
Winter (7)	12.33±1.07	7.37±0.54	7,982±505	4,422±209	6.95±0.15
Diff. (S−W)	−0.12±1.57	0.58±0.79	525±742	125±307	−0.54±0.23*

NOTE: In parenthesis, number of animals.
*Statistically significant at the 5% level.
**Statistically significant at the 1% level.
†Standard error.

Similar measurements on the adrenal glands of "outdoor summer" and "winter" rats revealed no difference in the size of the different zones, the number of fasciculata cells per unit of area, or the total number of fasciculata cells in the largest section of the gland. The diameter of the nuclei in the fasciculata zone, however, was greater ($P < 0.05$) in the "winter" rats. There was no difference between the two groups kept outdoors in the total volume of the adrenal or in the volume of the medulla, expressed in per cent of the total gland volume.

In both "indoor" and "outdoor" rats, the final body weight was less in the cold-exposed animals than in the controls (Table III). The differences

TABLE III

Statistical analysis of "in vitro" steroid production

	Indoor rats			
	30° C	6° C	Diff. 30° C–6° C	Outdoor rats
Body weight, g				
Summer	479±14† (12)	411±14 (12)	68±20**	454±14 (12)
Winter	443±13 (15)	413±14 (12)	30±19	363±14 (12)
Diff. (S−W)	36±19	−2±20		91±20**
Adrenal weight, mg				
Summer	43.2±2.3	52.0±2.3	−8.8±3.2**	51.8±2.3
Winter	36.7±1.4	60.4±3.8	−23.7±4.1**	44.2±2.1
Diff. (S−W)	6.5±2.8*	−8.4±4.4		7.6±3.1*
Steroids/hour/rat, μl				
Summer	7.43±0.62	6.33±0.30	1.10±0.69	8.12±0.66
Winter	6.33±0.40	7.13±0.45	−0.80±0.49	10.11±0.45
Diff. (S−W)	1.10±0.70	−0.80±0.48		−1.99±0.88*
Average adrenal weight for common body weight, 428 g				
Summer	39.7±2.5	53.2±2.3	−13.5±3.4**	50.0±2.3
Winter	35.7±2.0	61.4±2.3	−25.7±3.1**	48.7±2.6
Diff. (S−W)	4.0±3.1	−8.2±3.2**		1.3±3.6
Steroids/hour for common adrenal weight, 47.6 mg				
Summer	7.81±0.45	5.95±0.45	1.86±0.65*	7.76±0.45
Winter	7.28±0.46	6.02±0.53	1.26±0.79	10.46±0.45
Diff. (S−W)	0.53±0.61	−0.07±0.65		−2.64±0.65**

NOTE: In parenthesis, number of rats.
*Statistically significant at the 5% level.
**Statistically significant at the 1% level.
†Standard error.

between the two "outdoor" groups and between the "summer 30° C" and "6° C" groups were significant at the 1% level but the difference between the "winter 30° C" and "6° C" group fell short of the customary level of significance. No seasonal effect could be observed in the "indoor" rats (summer 30° C and winter 30° C rats), which reached about the same final body weight, and similarly for summer and winter 6° C rats; the difference between the two "30° C" groups was not significant.

While the adrenal glands were significantly larger in the "6° C" rats than in the "30° C" rats during both summer and winter, they were significantly smaller in the "winter outdoor" rats than in the "summer outdoor" ones. A similar seasonal effect was observed in the indoor groups kept at 30° C but not in the groups kept at 6° C.

When all groups were considered as a whole, there were indications of a linear relationship between adrenal weight and body weight. The coefficient of regression was estimated to be 0.069±0.020 mg/g. The residual variances were of about the same order in all groups.

When differences in body weight were taken into account, or when average adrenal weights were calculated for the average body weight of 428 g, differences in adrenal weights between the "summer" and "winter outdoor" groups and between the "summer" and "winter 30° C" groups disappeared, but the

difference between the two "6° C" groups became significant. On that basis, the weight of the adrenals of the rats at 6° C remained greater than that of the adrenals of the rats at 30° C.

Steroid Production in Vitro

There was no difference between the production and release of steroids by quartered adrenals taken from "indoor 6° C" or "indoor 30° C" rats. No seasonal effect was observed in the groups at 30° C or 6° C.

When each group was considered individually, a significant ($P < 0.01$) linear relationship between steroids/hour and adrenal weight could only be observed in the "winter 30° C" group. However, when all groups were considered as an aggregate, such a relationship seemed to hold for all groups. A common coefficient of regression of 0.087 ± 0.022/mg steroid/hour/mg of adrenal weight was then calculated.

The rates of steroid production per hour were also calculated for a common adrenal weight of 47.6 mg. In the "summer 30° C" group this rate perceptibly exceeded that of the "summer 6° C" rats ($P < 0.05$). It also appeared to do so in the "winter" animals, although this difference fell short of the usual statistical level of significance. In the "winter outdoor" rats the rate of steroid production exceeded that in the "summer outdoor" animals ($P < 0.01$).

Discussion

The hypertrophy of the adrenals observed in rats kept indoors at 6° C occurred during the first week of exposure and remained constant thereafter; this became evident after adrenal weights were adjusted to a common body weight (Fig. 1). Since previous results have shown, through indirect evidence, that the activity of the adrenal cortex continues to increase for at least 3 weeks (2) it appears quite evident that the increased secretory rate is not proportional to the change in size of the gland, at least in long-term experiments.

The increase in size of the adrenals in the 6° C acclimated rats was due exclusively to an increased number of fasciculata cells, the cells responsible for the production of most steroids. Incidentally, it might be noted that the size of the medulla was not affected by the prolonged cold exposure.

After 12 weeks of exposure to cold the in vitro production of steroids per animal from entire adrenals was not any greater than in the control (30° C) rats, although there was a greater number of fasciculata cells in the adrenals of the cold-acclimated rats. When differences in weight of the adrenals were properly taken into account by using the actual coefficient of regression to calculate the rate of secretion for a common adrenal weight, it appeared that the glands of the "6° C" rats were secreting at a slightly slower rate than the adrenals of the "30° C" rats. In other words, each fasciculata cell was secreting at a slower rate or the proportion of cells secreting at a normal rate was slightly less in cold-acclimated rats than in non-acclimated ones.

These in vitro measurements are in complete agreement with previous observations made by Héroux and Hart (2) and Rossiter and Nicholls (8). These authors showed, by means of indirect evidence, such as fall in eosinophils

after ACTH injection and P^{32} uptake after exposure for 2 hours to −5° C that after 2 to 3 months of exposure to 6° C or 5° C, the adrenal activity had returned to a normal level. A similar pattern seems to hold for the rate of steroid formation in vitro by adrenals taken from rats exposed to cold for 6 months or longer (Schönbaum, unpublished).

In the "outdoor winter" rats, the picture was completely different. There was no increase in the number of fasciculata cells or in any of the other zones and no hypertrophy of the total gland. Yet the total steroid production in vitro per rat was greater in the "winter" animals, particularly when the steroid production was calculated for a common adrenal weight. This means that each fasciculata cell was secreting at a faster rate after acclimatization (1) to cold.

The adrenal hyperactivity in "winter" rats can hardly be taken as a sign that these animals were not fully adapted since they have shown the same degree of resistance to cold, the same increased peak metabolism, the same reduction in shivering as the "indoor cold" acclimated rats (4), and the latter have just been shown to have a normal adrenal activity. Moreover, their increased pelage insulation (4) and the absence of peripheral cold injury (6) would suggest a better acclimatization to cold. Apparently similar degrees of adaptation to cold can exist with different requirements of adrenocortical hormones depending on the environmental conditions.

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COMPARISON BETWEEN SEASONAL AND THERMAL ACCLIMATION IN WHITE RATS

IV. MORPHOLOGICAL AND PATHOLOGICAL CHANGES[1]

O. HÉROUX AND J. S. CAMPBELL

With the technical assistance of DONNA WRIGHT

Abstract

White rats have been shown to develop a similar degree of resistance to cold whether exposed individually to a continuous cold temperature or exposed in groups of 10 to the fluctuating environmental conditions prevailing outdoors during the winter.

Under these two different types of cold exposure, the rats were observed to have a reduced muscle growth. However the enlargement of the adrenals, thyroids, pituitary, heart, and digestive tract and the reduction of the mesenteric and subcutaneous fat and of the pelt weight, which have repeatedly been found in the indoor cold-acclimated rats, did not take place in the "outdoor winter" rats. Therefore it is clear that increased resistance to cold can be brought about without some of the anatomical changes characteristically associated with acclimation to continuous cold exposure.

Cold stimulation outdoors was not sufficiently severe or of sufficient duration to produce renal lesions, hypertension, or cardiac lesions of any importance, although the animals were exposed to an average temperature of $-10°$ C which would certainly produce these lesions in indoor individually exposed rats.

Through numerous laboratory studies it has been shown that white rats individually exposed to a constant cold temperature can develop an important degree of resistance to cold (2). It has also been observed that cold acclimation is accompanied by an increased thyroid activity (1), a reduction in muscle growth (5), a reduction in the mesenteric and subcutaneous fat (4, 11), and hypertrophy of the liver, heart, kidney, digestive tract, and adrenal and thyroid glands (4). In addition, development of cold injury (10), hypertension, and renal and cardiovascular lesions are consequences of exposure for many months to temperature just above zero (12) or for just a few months to much colder temperatures such as $-1°$ C to $-4°$ C (3).

Although these changes have repeatedly been observed to develop under these cold exposure conditions, it has always been questionable whether the same degree of cold resistance can be developed without these changes.

Recently, it has been possible, by exposing white rats in groups of 10 to the fluctuating environmental conditions prevailing outdoors during the winter, to demonstrate that these animals can have a similar degree of cold resistance as the cold room acclimated rats without increased thyroid activity (7) and without any peripheral cold injuries (8).

This paper will describe the effect of outdoor cold exposure on muscle growth, organ weights, and blood pressure and on renal and cardiovascular tissues.

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada, and from the Department of Pathology, Faculty of Medicine, University of Ottawa, Ottawa, Canada.

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Experimental Procedures

The animals, environmental conditions, feeding, and housing were as described in the preceding papers (6, 8, 9). Briefly, the rats were kept for 3 months in individual cages at 30° C or 6° C or in groups of 10 outdoors during the summer from May to August or during the winter from October to January. Outdoor tests were made during three consecutive summers and three consecutive winters. Temperatures prevailing outdoors during these 3 years have been reported in the first paper (6).

The different methods used in this study have all been previously described: dissection and weighing of the organs in (4) and muscle analysis in (5). Blood pressures were measured by the indirect method (3), but for practical reasons, instead of keeping the animals in a wooden box during the measurements, they were wrapped in a towel.

In the 1955 summer and 1955–56 winter groups, a complete dissection was made and all the organs were weighed, while in the other groups, only liver, heart, kidneys, adrenals, and thyroids were weighed. In each group, only a certain number of rats were chosen at random for organ weighing, the others being used for different tests. Within seasons, the organ weights obtained during the 3 consecutive years were averaged.

Results

Body Weights

Although initial mean body weights for the different groups were not different, the final body weights (Table I) were consistently lower in the cold-

TABLE I

Final body weights

Summer			Winter		
		Outdoors			
1956	459 ± 13†	(12)	1955–6	387 ± 13	(12)
1957	400 ± 18	(6)	1956–7	345 ± 12	(14)
1958	454 ± 10	(20)	1957–8	367 ± 13	(11)
		Indoors			
30° C	479 ± 13	(12)	30° C	442 ± 13	(12)
6° C	411 ± 13	(12)	6° C	413 ± 13	(12)

NOTE: Figures in parenthesis indicate number of animals per group.
† = Standard error.

exposed groups than in their respective controls. The "30° C" rats were on the average heavier than the "6° C" ones; the difference attained the 1% level of statistical significance in the summer groups but fell short of the 5% level in the winter groups, and all the outdoor summer groups exceeded the winter ones, the differences being statistically significant at the 1% level. All were equally variable in respect to body weight of individuals but the averages for corresponding groups differed appreciably between years. Growth was retarded most during the winter 1956–57, which was the coldest of the three winters during which our experiments were performed.

Muscle Composition

As previously observed (5) and noted again in these experiments, the reduction in total growth in the indoor cold-exposed rats was partly due to a retarded growth of the muscle mass, as shown by the reduced water and protein content in the muscle mass of the "6° C" rats (Table II). The 56-g difference in muscle

TABLE II

Average component weights in muscle

	Indoor			Outdoor		
	30° C (winter controls)	6° C	Difference 30−6° C	Summer	Winter	Difference S−W
Body weight (g)	491 ± 14‡ (7)	384 ± 15 (6)	107 ± 21**	463 ± 11 (5)	411 ± 10 (4)	53 ± 15**
Muscle mass (g)	223 ± 6 (7)	166 ± 6 (6)	56 ± 8**	230 ± 7 (5)	194 ± 8 (4)	36 ± 10**
Water (g)	160 ± 5 (7)	121 ± 5 (6)	39 ± 7**	172 ± 5 (5)	142 ± 6 (4)	30 ± 8**
Protein (g)	50 ± 1 (7)	41 ± 1 (6)	9 ± 2**	56 ± 2 (5)	46 ± 2 (4)	10 ± 2**
Lipid‡ (g)	13 ± 1 (7)	4 ± 1 (6)	9 ± 2**	3 ± 1 (5)	6 ± 2 (4)	−3 ± 2
Soleus muscle (mg)				190 ± 9 (6)	121 ± 9 (6)	69 ± 13**
Water (mg)				139 ± 6 (6)	89 ± 6 (6)	50 ± 9**
Protein (mg)				42 ± 3 (6)	27 ± 3 (6)	15 ± 4**
Lipid† (mg)				9 ± 2 (6)	5 ± 2 (6)	4 ± 3

NOTE: Figures in parenthesis indicate number of animals per group.
†Includes lipid, carbohydrate, and ash.
‡ = Standard error.
**Difference statistically significant at the 1% level.

mass between the "6° C and the 30° C" rats accounts for only 52% of the total difference in body weight; according to previous results the rest of the difference in body weight can be attributed to a smaller mesenteric and subcutaneous fat reserve as well as to a smaller pelt weight.

A 53-g difference in body weight was found between four 1955–56 "winter outdoor" rats and six 1956 "summer outdoor" rats. In these animals, the muscle mass was 36 g smaller in the winter than in the summer rats; thus 68% of the difference in body weight can be accounted for by the difference in muscle mass. In the total muscle mass as well as in the soleus muscle, there was a significant reduction (at the 1% level) in water and protein content after exposure to the outdoor winter conditions. There was no statistically significant difference in lipid content of muscles between the two outdoor groups, while this muscle component was significantly reduced in the indoor cold-exposed rats.

Organ Weights

Weights of liver, heart, kidneys, thymus, visceral organs taken as a whole, fat, and pelt were with statistical significance correlated within groups with body weights of individual rats. When these intragroup regressions were used to adjust organ weights for differences in body weight, the indications were that liver, heart, kidney, adrenals, thyroid, and pituitary of indoor "6° C" rats were all significantly hypertrophied ($P < 0.01$) (Table III). Liver, heart, and kidney of "outdoor winter" rats also tended to be larger than their summer controls but the differences were much less than between the indoor groups. Moreover, while the difference in kidney weights was statistically significant only at the 5% level, the difference in heart weight did not reach this level of statistical significance. One striking difference in the effect of outdoor cold

TABLE III

Average organ regressed weights (g) for a common body weight of 418.3 g

	30° C (winter controls)			6° C			*P* for differences between 30° C and 6° C	Summer			Winter			*P* for differences between S and W
Liver	12.2	±0.5	(6)	18.7	±0.4	(6)	<0.01	13.1	±0.32	(14)	15.4	±0.33	(12)	<0.01
Heart	1.2	±0.05	(6)	1.6	±0.04	(6)	<0.01	1.3	±0.03	(14)	1.4	±0.03	(12)	n/s
Kidney	2.3	±0.09	(6)	3.6	±0.09	(6)	<0.01	3.2	±0.06	(14)	3.4	±0.06	(12)	<0.05
Spleen†								0.9	±0.05	(6)	0.7	±0.05	(6)	<0.05
Lungs†								2.3	±0.16	(6)	2.1	±0.16	(6)	n/s
Digestive tract plus pancreas†								15.5	±0.55	(6)	14.9	±0.55	(6)	n/s
Genitals†								7.8	±0.23	(6)	6.5	±0.23	(6)	<0.01
Thymus†								0.227	±0.04	(6)	0.157	±0.04	(6)	n/s
Adrenals	0.035	±0.002	(6)	0.061	±0.002	(6)	<0.01	0.049	±0.001	(28)	0.048	±0.001	(23)	n/s
Thyroid	0.009	±0.002	(6)	0.018	±0.001	(6)	<0.01	0.019	±0.001	(18)	0.019	±0.001	(10)	n/s
Pituitary†	0.011	±0.0006	(6)	0.014	±0.0006	(6)	<0.05	0.013	±0.0005	(6)	0.013	±0.0006	(6)	n/s
Visceral organs plus brain								45.2	±1.0	(6)	45.6	±0.9	(6)	n/s
Fat								32.9	±1.8	(6)	30.3	±1.4	(6)	n/s
Pelt								68.6	±1.9	(6)	66.3	±1.6	(6)	n/s

NOTE: Figures in parenthesis indicate number of animals per group.
†Absolute organ weight (not significantly correlated to body weight).

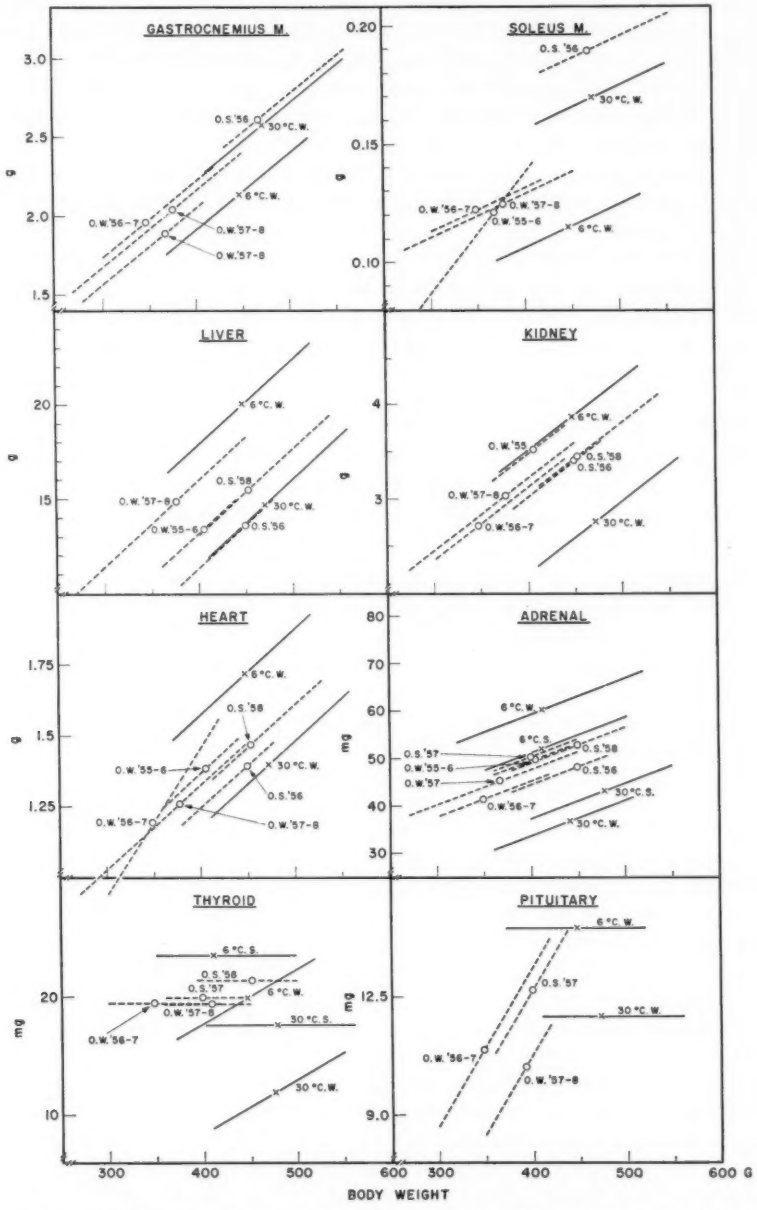

FIG. 1. Relationship between organ and body weights of "outdoor summer" (O. S.), "outdoor winter" (O. W.), 30° C summer (30° C S.), 30° C winter (30° C W.), 6° C summer (6° C S.), and 6° C winter (6° C W.) white Sprague-Dawley rats.

environment and indoor continuous cold conditions was in the endocrine glands; adrenal, thyroid, and pituitary weights remained normal after exposure to the winter conditions but were greatly hypertrophied in "6° C" rats.

The spleen and the genitals were smaller in the "winter" rats but lung, thymus, digestive tract, visceral organs taken together, fat, or pelt weights remained normal.

The interseasonal variations in organ weights and body weights as well as in the relationship between the two reduce the significance of the above comparisons. However, it is quite evident from Fig. 1 that the differences in organ weights relative to body weight between the summer and winter subgroups were much less than those between the indoor groups. For gastrocnemius and soleus muscles, the relation differed just as much between the two outdoor groups as between the two indoor groups.

Certain seasonal differences were observed in the "indoor rats". In the "6° C" rats the adrenals were larger during the winter than during the summer ($P<0.01$) and in both "30° C and 6° C" rats, the thyroids appeared to be larger during the summer than during the winter ($P<0.01$). Since different groups of rats had to be used at each season, it is uncertain whether these differences are a real seasonal effect or simply a batch effect.

Pathology and Blood Pressure

No pathological changes were found in any organ except in the heart where small foci of myocardial fibrosis existed in 66% of the "6° C" rats, in 33% of the "winter" rats, in 20% of the "summer" rats, and in none of the control "30° C" rats. These lesions were minimal and of questionable significance. The blood pressure was essentially normal in all groups; it was on the average 103 mm Hg for the "winter outdoor" rats, 94 mm Hg for the "summer outdoor" rats, and 100 mm Hg for both "30° C and 6° C" rats.

Discussion

Enlargement of the adrenals, thyroids, pituitary, heart, and digestive tract and reduction of the mesenteric and subcutaneous fat and of the pelt weight might be an invariable outcome of the continuous individual exposure to a constant cold temperature. It is clear, however, that these changes are not essential for increased cold resistance since they were not found in "outdoor winter" rats which were just as resistant to cold as the indoor "6° C" rats. These anatomical changes could be a consequence of an overstimulation due to the continuous exposure to cold.

Whether the other changes such as reduction in muscle growth, and therefore a reduction in total body weight, enlargement of the liver and the kidney, and reduction in weight of the genitals, which were observed under both types of cold exposure, are essential for increased cold resistance remains uncertain.

The normal weights of the endocrine glands indicate that under the outdoor conditions, cold stimulation was neither severe nor long enough at any given time to evoke an increased number of hormone-secreting cells.

Similarly, cold stimulation did not seem to be severe enough to produce renal lesions, high blood pressure, or cardiac lesions of any importance in the grouped

outdoor rats, although the environmental temperature was at times as low as −30° C and was on the average −10° C. Individual exposure indoors to less severe conditions (−1° C to −4° C) has been observed to produce hypertension within 2 months (3). This suggests that cold exposure experienced by the "outdoor" rats was so modified by huddling that its effect was not any greater than that experienced by the "6° C" rats which after 3 months had not developed any significant hypertensive diseases either.

Whether group exposure in a cold room continuously maintained at −10° C would offer similar protection and confer a similar degree of cold resistance to that seen in "outdoor" rats without producing pathological changes and enlargement of the visceral organs and of the endocrine glands, remains to be seen.

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EFFECT OF X-IRRADIATION ON NORMAL, HYPOTHYROID, AND HYPERTHYROID RABBITS[1]

P. V. VITTORIO, H. MARS, AND M. J. JOHNSTON

Abstract

The present study was undertaken to investigate the effect of a sublethal dose of X-irradiation on rabbits that had been made hyperthyroid with L-thyroxine-sodium and triiodothyronine or hypothyroid with Methimazole.

X-irradiation (400 r) administered on the 25th day after drug treatment was started caused an initial sharp loss in weight in control rabbits and in hyperthyroid rabbits. However, by the 22nd day after X-irradiation the weight of the irradiated control group was comparable with that of the non-irradiated controls, but the weight of the hyperthyroid group was still below that of the non-irradiated hyperthyroid group. The irradiated hypothyroid group never showed a loss in weight. They gained weight initially but at a slower rate than the non-irradiated hypothyroids, and by the 22nd day after X-irradiation were comparable to the non-irradiated hypothyroid animals. X-Irradiation produced an initial decrease in the hematocrit value of the three groups under study, and the hyperthyroid animals showed the greatest drop in hematocrit value. Twenty-two days after X-irradiation only the controls had returned to their original hematocrit value prior to irradiation. After X-irradiation, the albumin/globulin ratio was decreased in all three groups with the effect greatest on the hyperthyroid animals, less on the controls, and least on the hypothyroid animals. The A/G ratio of the hypothyroid group returned to normal more rapidly than did that of the controls. The A/G ratio of the hyperthyroid group was still much below normal 22 days after X-irradiation.

In terms of mortality the hyperthyroid group were more radiosensitive than the control or hypothyroid group.

Introduction

Two reports (1, 2) have shown that high doses of thiourea protected mice from the lethal effects of roentgen ray irradiation. Both these reports dealt with dosages of thiourea that were within the toxic range. Haley *et al.* showed that thiourea (3) or thiouracil (4), antithyroid drugs, fed to rats in a non-toxic dose did not protect rats from the lethal effects of roentgen ray irradiation even though the animals became hypothyroid. They also pointed out that hyperthyroidism, produced by the subcutaneous injection of thyroxine into rats, increased the mortality rate and in one experiment the total mortality of roentgen ray irradiated rats (3). Experiments to correlate radiosensitivity with metabolic state for the most part have consisted of the determination of the LD_{50} of animals whose metabolic state had been altered by drug therapy or surgery.

The present study was carried out to investigate the effect of a sub-lethal dose of X-irradiation on rabbits that had been made hyperthyroid or hypothyroid by the use of drugs, administered orally in non-toxic doses. Radiosensitivity was measured in terms of effect on total body weight, hematocrit value, and albumin/globulin (A/G) ratio.

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Methods

Eighteen white male rabbits of approximately the same age (about six months) and weight (2–3 kg) were used in this study. They were divided into three groups of six, and each rabbit was caged individually. One group was made hypothyroid (hypometabolic) by feeding each rabbit 1.2 mg per day of Methimazole (Tapazole).

A second group was made hyperthyroid (hypermetabolic) by feeding each rabbit a mixture of L-thyroxine-sodium, 30.0 μg per day, and triiodothyronine, 2 μg per day. A measured volume of the required mixture was forced down the oesophagus of the rabbit, by means of a syringe, daily for the duration of the experiment. The animals in the third group were maintained as controls. All three groups were supplied with food and water ad libitum.

The above treatment was carried out for 25 days, then half (three) of the rabbits in each group were irradiated. After X-irradiation all animals were returned to their cages and feeding and treatment with drugs were continued in the same manner as before X-irradiation.

X-Irradiation was carried out using a Mueller X-ray machine operating at 300 kv, 5 ma, delivering an air dose of 17.7 r per minute at a point arbitrarily chosen as the center of the animal. The filtration employed was Al 0.137 g/cm^2 (one sheet) and Cu 0.888 g/cm^2 (two sheets). A total dose of 400 r was administered to each rabbit. (The LD_{50} 30 days for the rabbit is 800 r.)

About four milliliters of blood was taken from the marginal ear vein of each rabbit. Each sample was permitted to coagulate and the exuded serum was separated by centrifugation and dialyzed against 0.9% NaCl solution for at least 48 hours at 4° C. The dialyzed serum was then used for protein analysis by paper electrophoresis.

Hematocrit values were determined at each bleeding using heparinized capillary tubes which were sealed at one end in a flame and centrifuged in an International Micro-Capillary Centrifuge Model M.B.

Serum proteins were separated using paper electrophoresis. Electrophoretic separation was carried out using the Spinco Model R system as outlined in Spinco Technical bulletin No. TB 6052A. The serum proteins on the paper strips were stained using bromophenol blue dye in alcoholic solution as outlined in Spinco Technical Bulletin No. TB 6050A and scanned with a Model RB Spinco Analytrol fitted with two 500-mμ interference filters.

Results

The non-irradiated groups that were treated with drugs throughout the 50-day experimental period suffered no fatalities so that one may conclude that non-toxic doses of drugs were administered.

Figure 1 shows the average percentage change in total body weight for the non-irradiated and irradiated groups. The average weights of the hypothyroid, hyperthyroid, and control groups at the beginning of the experiment were 2473, 2477, and 2457 g respectively. After 50 days of drug treatment and no irradiation the hypothyroid groups showed a 3% greater increase in weight than

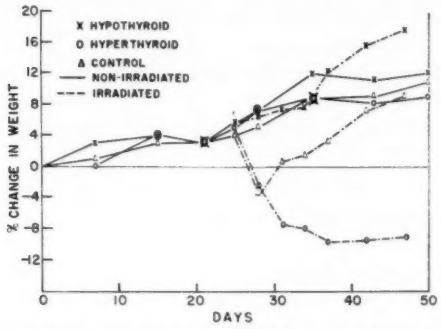

FIG. 1. The effect of X-irradiation on the total body weight of control, hypothyroid, and hyperthyroid rabbits.

the hyperthyroids and 1% greater increase than the controls. During the first 9 days after X-irradiation the hypothyroids showed less than half the increase in weight shown by the non-irradiated hypothyroid group for the same time period. However, from the 10th to the 22nd day the irradiated hypothyroids showed a steady increase in weight and they reached a slightly higher weight than did the non-irradiated hypothyroids. The irradiated controls showed a rapid decrease up to the third day and then began to increase in weight. About the 12th day they were back to their original weight at the time of irradiation and by the 22nd day they were comparable to the non-irradiated controls. The irradiated hyperthyroid group lost weight steadily until the 12th day and by the 22nd day was still more than 10% below the original weight at time of irradiation.

In summary, X-irradiation affects weight increase more in hyperthyroid animals than in control animals and the latter are affected more than hypothyroid animals.

Figure 2 shows the percentage change in hematocrit value for the non-irradiated and irradiated groups. The average hematocrit value for each group

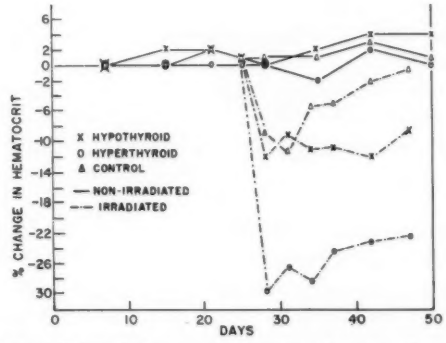

FIG. 2. The effect of X-irradiation on the hematocrit value of control, hypothyroid, and hyperthyroid rabbits.

at the beginning of the experiment was 41, and rabbits within the same group showed a $\pm 3\%$ variation in hematocrit value. Since the hematocrit values of the non-irradiated hyperthyroid and hypothyroid groups never varied much more than the controls it appeared that the drug treatment used to bring about the changes in metabolic state did not cause any significant change in hematocrit value.

Two days after X-irradiation the control, the hypothyroid, and hyperthyroid groups showed a decrease in hematocrit value. The hyperthyroids showed the greatest decrease; 22 days after X-irradiation the hematocrit value was about 27% lower than the value just prior to irradiation. Two to six days after X-irradiation the value for the hypothyroid and control groups decreased by about 10% but the control group showed the more rapid recovery. Twenty-two days after X-irradiation the hematocrit value of the control group was back in the normal range but the hematocrit value of the hypothyroid group was still about 10% lower than the value prior to X-irradiation.

Figure 3 shows the average serum A/G ratio for the irradiated and non-irradiated groups. The average A/G ratio for each group at the beginning of

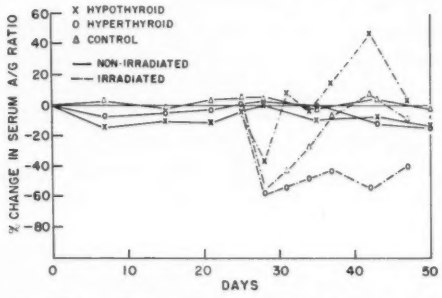

FIG. 3. The effect of X-irradiation on the serum A/G ratio of control, hypothyroid, and hyperthyroid rabbits.

the experiment was 1.7 and rabbits within the same group showed a $\pm 5\%$ variation in serum A/G ratio. During the experimental period (50 days) the A/G ratio of the non-irradiated groups never decreased by more than about 15% of the original value. Two days after X-irradiation the A/G ratio of all three groups decreased in the order hyperthyroids > the controls > the hypothyroids. The value for the hypothyroid group appeared to return to the pre-irradiation value by about the 6th day after X-irradiation and the controls by about the 12th day.

It is interesting to note that on the 17th day after X-irradiation the hypothyroids showed an increased A/G ratio which returned to the pre-irradiation value on the 22nd day. The decreased A/G ratio of the irradiated hyperthyroid group did not show much tendency to recover, and 22 days after X-irradiation the A/G ratio was still about 40% lower than the value prior to irradiation.

An experiment was carried out to study the effect of 500 r of X-irradiation on rabbits in different metabolic states that had received drug treatment for

49 days prior to irradiation. The results are shown in Table I. The hyperthyroid group all died within 1 day after X-irradiation but the control and hypothyroid groups suffered no casualties and were still alive 30 days later.

TABLE I

The effect of X-irradiation on rabbits in different metabolic states

Daily oral treatment	Treatment time, days	Metabolic state	X-Irradiation, roentgens	No. of animals used	No. of deaths 1 day later
Water	49	Control	500	3	None
Methimazole	49	Hypothyroid	500	3	None
{L-Thyroxine-sodium {Triiodothyronine	49	Hyperthyroid	500	3	3

Discussion

In terms of mortality the hyperthyroid group was the most sensitive to radiation, since all of the hyperthyroid animals died in 1 day when exposed to 500 r of X-irradiation. The control and hypothyroid groups suffered no casualties when exposed to the same amount of X-irradiation. However, only one dosage (500 r) was used, so one cannot predict that the controls and hypothyroids would have the same survival rate at different dosages. Experiments to answer this question are already under way.

When radiosensitivity was measured in terms of total body weight, hematocrit value, and blood serum A/G ratio, the three groups under study reacted differently to these indices of response. Initially, the hypothyroid and control groups are affected by X-irradiation (400 r) but each to a different degree, and both groups appeared to recover. Recovery appeared to be well under way 6 to 8 days after X-irradiation. The hyperthyroid group was affected to a much greater degree than either of the other two groups and 22 days after X-irradiation still had not recovered. From these results one might have predicted that an increase in the radiation dose would produce death in the hyperthyroid group before it produced death in the hypothyroid and control groups.

From the results obtained in this study it appears reasonable to conclude that the metabolic state plays an important role in determining the radiosensitivity of rabbits.

Acknowledgment

The authors wish to thank Mrs. W. P. Spence for her interest and assistance during the course of this investigation.

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BIOSYNTHESIS OF PHENYLALANINE AND TYROSINE IN YOUNG WHEAT AND BUCKWHEAT PLANTS[1]

O. L. GAMBORG AND A. C. NEISH

Abstract

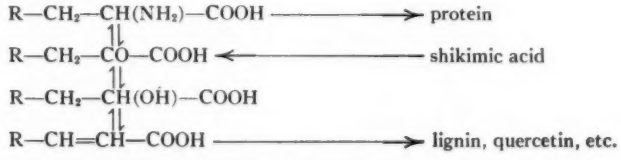
Several C^{14}-labelled compounds were fed to shoots excised from young plants of *Triticum vulgare* Vill. or *Fagopyrum tataricum* (L.) Gaertn. The free and bound phenylalanine and tyrosine were isolated after a metabolic period of 6 hours and their C^{14} content measured. Shikimic acid was superior to glucose and acetate as a precursor of both phenylalanine and tyrosine. Phenyllactic acid and phenylpyruvic acid were easily converted to free phenylalanine and were incorporated into bound phenylalanine as readily as was free phenylalanine itself. Similarly, *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylpyruvic acid were converted preferentially to tyrosine. There was some conversion of phenyl compounds to *p*-hydroxyphenyl compounds but little evidence of the reverse reaction. Radioactive carbon fed as cinnamic, dihydrocinnamic, or *p*-coumaric acids was not incorporated into either phenylalanine or tyrosine.

Introduction

Previous studies have shown that both shikimic acid and phenylalanine are good precursors of lignin (1) and quercetin (2) in higher plants. These results suggest that phenylalanine may be formed in plants by the same route established by Davis and his collaborators (3, 4) in bacteria. This hypothesis was strengthened when it was found that shikimic acid was easily converted to both phenylalanine and tyrosine in *Salvia splendens* Sello. (5). It is also supported by the recent observation that quinic acid can be converted to shikimic acid, phenylalanine, and tyrosine in rose cuttings (6).

Phenylalanine has been found to act as a precursor of a number of phenylpropanoid compounds in plants. These include lignin (1, 7, 8, 9, 10, 11), quercetin (2), coniferin (11), *p*-coumaric acid (12), caffeic acid (12, 13, 14), and ferulic acid (12). Tyrosine is a good precursor of lignin in the Gramineae but has not yet been found to be converted easily to lignin by a species belonging to any other plant family (7, 10). On the basis of feeding experiments with C^{14}-labelled compounds of the phenylpropanoid type a scheme has been suggested for the interconversion of simple C_6C_3 compounds in plants (10, 12).

The experiments presented in this paper were designed to test the hypothesis that shikimic acid is converted to phenylpropanoid compounds in plants by the following reactions:



where R = phenyl or *p*-hydroxyphenyl.

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In previous work this hypothetical metabolic pool has been tested mainly in the direction of formation of lignin and other non-nitrogenous aromatic substances. However *p*-hydroxyphenylpyruvic acid was shown to form tyrosine in buckwheat (10). It has also been observed that cinnamic acid (C_6H_5—CH=CH—COOH) is not converted readily to phenylalanine or tyrosine in *Salvia splendens* (5) and this suggests that at least one of the steps shown above is irreversible.

In the present work shoots from young plants were fed the precursors depicted above (labelled with C^{14}) and the C^{14} contents of the free and bound phenylalanine and tyrosine were measured. This was done to test the proposed scheme in the reverse direction from the studies on which it was originally based (10, 12). Young plants were used because they would be expected to carry on a rapid synthesis of protein and to form relatively little lignin. One of the species selected for this study (i.e. wheat) can convert both phenylalanine and tyrosine to lignin while the other species (buckwheat) cannot utilize tyrosine for lignin formation (7).

Experimental

Cultivation of Plants

Young plants of Thatcher wheat (*Triticum vulgare* Vill.) and of Tartarian buckwheat (*Fagopyrum tataricum* (L.) Gaertn. var. C.D. 4251) were grown in subirrigated gravel culture, using a modified Hoagland solution (15). Light was supplied by a combination of "cool white" fluorescent tubes and incandescent bulbs at an intensity of about 14,000 lux. The day length was 18 hours and the temperature was 22° C during the day and 18° C at night.

C^{14}-Labelled Compounds

Sodium acetate-1-C^{14}, D-glucose-1-C^{14}, and L-tyrosine-G-C^{14} (i.e. generally labelled) were purchased from Atomic Energy of Canada Limited. The *p*-hydroxyphenylpyruvic acid-β-C^{14} was prepared by the method of Billek and Herrmann (16) and a portion of it was reduced to *p*-hydroxyphenyllactic acid-β-C^{14} by sodium amalgam (17). The preparation of shikimic acid-G-C^{14} (5), *p*-coumaric acid-β-C^{14} (8), phenylpyruvic acid-β-C^{14} (18), and the other C^{14}-labelled compounds (8, 9) is described elsewhere.

Administration of C^{14}-Labelled Compounds

The compounds were dissolved in 0.5–1.0 ml of water in a 2.5×11 cm test tube. The acids were brought into solution by adding an equivalent amount of sodium hydroxide, when necessary. The plants were pulled from the gravel and the roots excised under water. About 40–50 wheat plants and 20 buckwheat plants were used in each experiment. The shoots were then allowed to absorb the labelled solution through the cut ends of the stems as described previously (5).

The wheat plants were placed in a chamber at 21° C and received light from "cool white" fluorescent tubes (about 5000 lux). In order to prevent wilting the buckwheat shoots were put in a plastic compartment set over a basin of water in the greenhouse. The plants were kept moist by spraying occasionally

with a fine mist of water. The light intensity was about 5000 lux and the temperature ranged from 22–25° C. During the 6-hour metabolic period the shoots absorbed the solutions and several 1–2 ml washings of distilled water.

Extraction of the Plant Material

At the end of the metabolic period the shoots were cut up and extracted with hot 80% ethanol (3 ml/1 g fresh weight) in a VirTis homogenizer. The mixture was filtered on a Buchner funnel and the residue washed with hot 80% ethanol followed by acetone and diethyl ether. The residue was saved for isolation of the "bound" amino acids. The filtrate was evaported to dryness by an air stream at room temperature and then dissolved in a small volume of hot water. Celite Analytical Filter Aid was added to make a paste and then more hot water was added. The mixture was heated 20 minutes on a steam bath, and filtered with suction. The filter cake was washed several times with hot water and discarded. The filtrate was evaporated to a volume of 20–30 ml by an air stream at room temperature and used for isolation of the "free" amino acids as described below.

Isolation of Free Phenylalanine and Tyrosine

The solution obtained above was put through a 1.2×15 cm column of Amberlite IR-120-H ion exchange resin. The column was washed with distilled water and the eluate and washings were discarded. The amino acids were then eluted from the column with *M* ammonium hydroxide and the eluate evaporated to dryness by an air stream at room temperature.

The residue was dissolved in 1–2 ml of 0.5 *N* acetic acid and put on to a 2×55 cm column of Dowex-1-acetate. The phenylalanine and tyrosine were eluted in separate fractions by 0.5 *N* acetic acid as described previously (19, 5). The fractions were evaporated to dryness and their purity tested by paper chromatography using *n*-butanol:acetic acid:water (4:1:1.8, v/v) as the developing solvent. This showed each of the fractions to contain only one ninhydrin-positive substance which had the same mobility as the amino acid in question.

The amount of each amino acid in an aliquot was determined by the quantitative ninhydrin method of Moore and Stein (20) and C^{14} was measured on the remainder of the material. In experiments 4, 5, 6, 9, 10, and 11 (see Table I) the free phenylalanine was diluted with 1–3 parts of accurately weighed carrier and recrystallized twice, as described below, before C^{14} analysis. In experiments 13 and 15 the free tyrosine was also recrystallized with added carrier. The results are reported on a carrier-free basis.

Isolation of Bound Phenylalanine and Tyrosine

The residue from the alcohol extraction of the plant material (1.0–1.5 g) was mixed with 40 ml of 6 *N* hydrochloric acid in a sealed glass tube and heated 24 hours at 100° C. The hydrolyzate was filtered and the filtrate evaporated to dryness by an air stream at room temperature. The amino acids of this mixture were isolated by adsorption on Amberlite IR-120-H and further purified on a Dowex-1-acetate column as described above. This gave samples of phenylalanine and tyrosine which appeared to be pure when tested by paper chromatography. The amount of each amino acid was measured quantitatively (20) on

TABLE I

Biosynthesis of phenylalanine and tyrosine from C^{14}-labelled compounds in young wheat shoots*

Expt. No.	Compound administered	Per gram dry weight of plant: Precursor absorbed		Per gram dry weight of plant: μM isolated				Specific activity, mμc/mM			
				Phenylalanine		Tyrosine		Phenylalanine		Tyrosine	
		μM	mμc	Free	Bound	Free	Bound	Free	Bound	Free	Bound
1	Shikimic acid†	38.2	1500	4.1	54.8	1.9	26.1	26,300	380	16,700	420
2	Dihydrocinnamic acid‡	39.4	1660	1.7	52.0	1.6	28.1	188	5.6	31.9	1.8
3	Cinnamic acid‡	39.8	3660	3.9	63.5	1.3	23.5	427	0.9	8.4	2.0
4	(−)-Phenyllactic acid‡	38.2	3970	16.2	51.5	2.0	31.0	53,800	1970	23,300	350
5	Phenylpyruvic acid§	20.4	1930	14.0	61.0	1.6	28.2	31,200	1420	4300	250
6	L-Phenylalanine§	40.6	2520	27.1	78.5	2.4	33.2	60,400	1100	5200	15.6
7	Sodium acetate-1-C^{14}	12.3	1190	3.0	43.6	1.5	23.4	211	16.9	83	5.0
8	D-Glucose-1-C^{14}	10.8	3080	2.7	35.7	1.3	16.7	3800	188	1480	182
9	(−)-Phenyllactic acid‡	13.2	1500	7.5	50.4	1.7	27.8	53,500	1840	10,300	315
10	Phenylpyruvic acid§	11.4	1070	6.6	41.1	1.1	19.2	40,000	1480	9800	320
11	L-Phenylalanine§	11.3	705	8.7	51.6	1.2	21.5	40,000	1080	4800	31.4
12	*p*-Coumaric acid§	13.2	412	3.8	60.0	1.7	25.2	25.1	0	25.5	0
13	DL-*p*-HPLA§	12.3	1020	1.2	48.7	3.3	22.3	155	0	48,600	1350
14	*p*-HPPA§	12.0	970	Lost	46.4	Lost	19.5	—	0	—	1450
15	L-Tyrosine†	11.4	1070	3.2	48.7	7.7	19.9	1080	17.1	59,100	1580

*Experiments 1–6 were carried out with 50 shoots (per experiment) from one group of seedlings 2 weeks old (dry weight, 1.1–1.4 g) and experiments 7–15 with 40 shoots (per experiment) from another group 3 weeks old (dry weights, 3.6–4.2 g). The metabolic period was 6 hours.

†Generally labelled with C^{14}.

‡Labelled with C^{14} in the ring and β-carbons.

§Labelled with C^{14} in β-carbon only.

Abbreviations: HPLA = hydroxyphenyllactic acid; HPPA = hydroxyphenylpyruvic acid.

an aliquot and the main fraction subsequently diluted with a known proportion of carrier (1–3 parts) and recrystallized to constant specific activity. Tyrosine was recrystallized from hot water. Phenylalanine (35 mg) was dissolved in a mixture of 0.4 ml of water and 0.3 ml of pyridine by heating. More pyridine was then added dropwise until the solution became slightly turbid. The mixture was cooled at 3° for about 20 hours and the crystals collected by suction in a sintered glass filter and dried in a vacuum desiccator to constant weight.

C^{14} Determinations

The samples were converted to carbon dioxide by wet combustion and the activity measured in a 250-ml ion chamber by the Dynacon Electrometer (Nuclear-Chicago Corporation). This method can detect 0.1 mμc of C^{14}.

Results

Tables I and II show the results obtained with two different groups of wheat plants. The figures in Table II were calculated from the data in Table I. This calculation was necessary because of variations in the specific activities of the precursors and in the yields of products. Since the amount of free amino acid isolated was small and the experimental errors subsequently are comparatively large, the data on the free amino acids should be considered as being only semiquantitative. Table II also gives the structural formulae of compounds referred to by name in Table I.

TABLE II

Efficiency of C^{14}-labelled compounds as precursors of phenylalanine and tyrosine in young wheat shoots

Expt. No.	Compound administered†	% of C^{14} fed found in:*			
		Phenylalanine		Tyrosine	
		Free	Bound	Free	Bound
1	Shikimic acid	7.20	1.38	2.08	0.73
2	R—CH_2—CH_2—COOH	0.019	0.002	0.003	0.003
3	R—CH=CH—COOH	0.045	0.002	Trace	0.001
4	R—CH_2—CH(OH)—COOH	22.0	2.56	1.17	0.27
5	R—CH_2—CO—COOH	22.6	4.50	0.36	0.36
6	R—CH_2—CH(NH_2)—COOH	64.8	3.42	0.50	0.021
7	CH_3COONa	0.053	0.062	0.010	0.098
8	D-Glucose	0.32	0.22	0.062	0.098
9	R—CH_2—CH(OH)—COOH	26.7	6.16	1.17	0.58
10	R—CH_2—CO—COOH	24.7	5.69	1.01	0.57
11	R—CH_2—CH(NH_2)—COOH	49.4	7.92	0.82	0.096
12	R′—CH=CH—COOH	0.023	Nil	0.01	Nil
13	R′—CH_2—CH(OH)—COOH	0.018	Nil	15.7	3.01
14	R′—CH_2—CO—COOH	Lost	Nil	Lost	3.28
15	R′—CH_2—CH(NH_2)—COOH	0.32	0.08	42.5	2.94

*Calculated from data in Table I, i.e.

$$\frac{100 \times \mu M \text{ of amino acid isolated} \times m\mu c/\mu M \text{ of amino acid}}{m\mu c \text{ precursor absorbed}} = \% \text{ of } C^{14} \text{ fed found in amino acid.}$$

†Position of label and trivial name is given in Table I.
R = phenyl; R′ = *p*-hydroxyphenyl.

The percentage conversion figures (Table II) show that shikimic acid was converted more readily than glucose to phenylalanine and tyrosine. On the other hand sodium acetate was inferior to glucose. Cinnamic acid, dihydro-

TABLE III

Biosynthesis of phenylalanine and tyrosine from C^{14}-labelled compounds in young buckwheat shoots*

Expt. No.	Compound administered	Per gram dry weight of plant: Precursor absorbed		Per gram dry weight of plant: μM isolated				Specific activity, mμc/mM			
				Phenylalanine		Tyrosine		Phenylalanine		Tyrosine	
		μM	mμc	Free	Bound	Free	Bound	Free	Bound	Free	Bound
16	D-Glucose-1-C^{14}	10.7	460	1.1	60.3	0.73	35.7	1620	49	111	18.3
17	Shikimic acid†	11.2	440	2.3	48.6	1.2	28.7	17,200	860	11,000	723
18	Cinnamic acid§	9.3	870	1.1	52.3	0.78	32.0	78	1.5	95	0
19	(−)-Phenyllactic acid‡	9.8	1050	2.2	69.4	0.85	40.2	31,540	1410	3020	51.8
20	Phenylpyruvic acid§	8.2	760	2.0	63.0	1.0	40.0	27,870	1810	8400	194
21	L-Phenylalanine§	10.6	675	2.4	66.2	0.73	39.0	37,600	1170	337	24.4
22	*p*-Coumaric acid§	9.6	290	0.75	59.7	0.88	41.4	84	9.9	30	2.9
23	DL-*p*-HPLA§	8.6	710	1.2	60.3	0.90	38.4	10.9	0.84	5370	560
24	*p*-HPPA§	8.3	670	0.91	51.0	0.63	32.2	220	6.5	10,420	1510
25	L-Tyrosine†	8.6	1670	1.7	48.3	4.5	28.4	1510	3.2	95,000	6900

*Shoots (20 per experiment) were taken from a batch of plants 19 days old. Dry weight was 2.1–3.0 g per experiment. The metabolic period was 6 hours.
†Generally labelled with C^{14}.
‡Labelled with C^{14} in ring and β-carbons.
§Labelled with C^{14} in β-carbon only.
Abbreviations: HPLA = hydroxyphenyllactic acid; HPPA = hydroxyphenylpyruvic acid.

cinnamic acid, and *p*-coumaric acid were not converted to the amino acids to any appreciable extent. The most striking changes were the ready conversion of phenyllactic and phenylpyruvic acid to phenylalanine. This can be seen in the increase of free phenylalanine when these compounds were fed (Table I) as well as in the percentage of C^{14} incorporated (Table II). There was little difference between these compounds; both were converted equally well to phenylalanine. Actually the incorporation of C^{14} into the bound phenylalanine (i.e. into protein) was about the same whether phenyllactic acid, phenylpyruvic acid, or phenylalanine itself was fed. There was an appreciable conversion of phenyllactic acid and phenylpyruvic acid to tyrosine but this was low compared with their conversion to phenylalanine, and phenylalanine itself was converted to tyrosine at a relatively low rate. Similarly *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylpyruvic acid were just as good precursors of bound tyrosine as was tyrosine itself. The conversion of *p*-hydroxyphenyl compounds to phenylalanine was very slow if it occurred at all.

Tables III and IV show the results obtained with young buckwheat plants. The free amino acid pools in buckwheat were smaller than in wheat, and the

TABLE IV

Efficiency of C^{14}-labelled compounds as precursors of phenylalanine and tyrosine in young buckwheat shoots

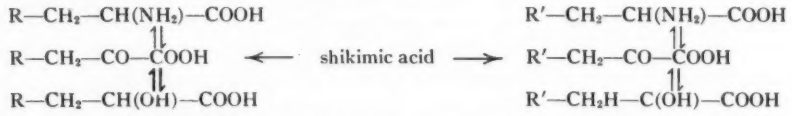
Expt. No.	Compound administered†	% of C^{14} fed found in:*			
		Phenylalanine		Tyrosine	
		Free	Bound	Free	Bound
16	D-Glucose	0.39	0.64	0.018	0.142
17	Shikimic acid	9.00	9.48	3.00	4.73
18	R—CH=CH—COOH	0.01	0.01	0.001	Nil
19	R—CH_2—CH(OH)—COOH	6.60	9.30	0.25	0.20
20	R—CH_2—CO—COOH	7.35	15.0	1.10	1.02
21	R—CH_2—CH(NH_2)—COOH	13.4	11.5	0.036	0.14
22	R′—CH=CH—COOH	0.02	0.20	0.01	0.04
23	R′—CH_2—CH(OH)—COOH	0.002	0.007	0.68	3.03
24	R′—CH_2—CO—COOH	0.03	0.05	0.98	7.25
25	R′—CH_2—CH(NH_2)—COOH	0.15	0.01	25.5	11.7

*Calculated from data in Table III (see footnote to Table II).
†The position of the label and the trivial name is given in Table III.
R = phenyl; R′ = *p*-hydroxyphenyl.

ratio of C^{14} in the bound amino acids to that in the free amino acids was higher in buckwheat. Shikimic acid was converted to phenylalanine and tyrosine quite readily, being superior to glucose in this respect. In general the results correspond to those obtained with wheat. The lactic acid and pyruvic acid derivatives were converted readily to the corresponding alanine derivatives, while cinnamic acid and *p*-coumaric acid were not. There appears to have been an appreciable conversion of phenylpyruvic acid to tyrosine (see experiment 20) but there was little conversion of the *p*-hydroxyphenyl compounds to phenylalanine.

Discussion

The results obtained above support the view that the following reactions occur in young wheat and buckwheat plants:



where R = phenyl and R′ = *p*-hydroxyphenyl.

Formation of the amino acids from the keto acids would be expected to occur by transamination and it has already been shown that the formation of phenylalanine from phenylpyruvic acid and glutamic acid can be catalyzed by a plant enzyme preparation (21). There are no enzymes known which specifically catalyze the interconversion of the keto and hydroxy acids shown above, but it has been found that lactic acid dehydrogenase is not highly specific and indeed it will catalyze the reduction of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid although at a relatively low rate (22). It is quite possible that these plant tissues contained enough lactic acid dehydrogenase to carry out the changes observed.

Shikimic acid appears to act as a common precursor of phenylalanine and tyrosine as would be expected if plants synthesize these amino acids by the same pathways as bacteria do. The amounts of C^{14} incorporated from shikimic acid into bound phenylalanine and tyrosine are approximately in the same ratio as the amounts of phenylalanine and tyrosine isolated. This would be expected if both amino acids were formed only by the shikimic acid pathway and assuming the cuttings synthesize these amino acids at the same relative rates as are indicated by their composition.

On the other hand phenylpyruvic acid is converted to phenylalanine much more readily than it is to tyrosine. This makes it seem unlikely that much tyrosine is formed from phenylpyruvic acid in plants, although some synthesis by this route may occur. It appears that the major part of the tyrosine originates from *p*-hydroxyphenylpyruvic acid which is formed directly from prephenic acid as has been shown in *Escherichia coli* (23, 29).

There is some evidence, however, of the conversion of phenyl compounds to *p*-hydroxyphenyl compounds (Tables II and IV). This was more noticeable with phenylpyruvic acid and phenyllactic acid than with phenylalanine. Apparently the hydroxylation systems in plants differ from that isolated by Mitoma and Leeper (24) from rat liver. Their system carried out a specific conversion of phenylalanine to tyrosine and would not hydroxylate phenylpyruvic acid. Attempts to find this specific phenylalanine hydroxylase in microorganisms have given negative results and even in animals it appears to be found only in the liver (24, 25). It is possible that phenylalanine hydroxylase is confined to organisms which are unable to synthesize the aromatic amino acids. The hydroxylation of phenyl compounds in plants may occur preferentially with phenylpyruvic acid. In this connection it should be mentioned that a less specific hydroxylation system has been found in animal tissues (26).

Furthermore, Marion and co-workers (27, 28) have shown that hordenine can be formed in barley seedlings from either phenylalanine or tyrosine and they have concluded that phenylalanine is converted to tyrosine. Their results do not exclude the sequence:

phenylalanine ⟶ phenylpyruvic acid ⟶ *p*-hydroxyphenylpyruvic acid ⟶ tyrosine.

The results of this investigation lend some support to suggested schemes (10, 12) for the interconversion of phenylpropanoid compounds (see Introduction). There is no evidence, however, for reversal of the reaction:

$$R{-}CH_2{-}CH(OH){-}COOH \longrightarrow R{-}CH{=}CH{-}COOH$$

(where R = phenyl or *p*-hydroxyphenyl) but the other reactions can occur in the reverse direction from that studied previously.

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REMOVAL OF STRONTIUM AND CESIUM FROM MILK[1]

B. B. MIGICOVSKY

Abstract

Radiostrontium can be removed from milk by stirring it with a cation exchange resin saturated with calcium. Successive treatment of milk with the resin will remove 95% of added strontium. Strontium which enters milk via the physiological mechanism is also removed by this process. Treatment of milk with a calcium-saturated resin raises the calcium content and reduces the potassium and sodium content of the milk.

Resin that is equilibrated with a solution of $CaCl_2$, KCl, and NaCl which has the same cation proportions as does milk is effective in removing strontium and cesium and does not alter the calcium, potassium, or sodium content of the milk.

Introduction

The appearance of radioactive isotopes of strontium and cesium in food has generated considerable concern. There is complete agreement on the fact that these isotopes constitute a hazard to health. The controversy rages around the quantitative aspects of this hazard.

Although present levels of Sr^{90} and Sr^{89} may be considered relatively innocuous, it is within the realm of possibility that they will continue to rise. It becomes important, therefore, to reduce the hazard by restricting the entrance of these isotopes into the food and by removing them before the food is consumed.

One of the major sources of the strontium isotopes in the human diet is milk, and this creates a particular hazard for the young. It is likely therefore that a method whereby we can reduce or eliminate the strontium isotopes from milk, without altering the milk, could possibly be of value.

Ion exchange resins have been used for several years to separate strontium from other elements but the employment of cation exchange resins in the hydrogen form to remove strontium from milk alters the milk irreversibly.

It has been repeatedly observed by investigators studying bone metabolism that isotopes of calcium and strontium are removed from blood very quickly and appear in the skeleton (1, 2). The heteroionic exchange with skeletal calcium accounts in large part for this rapid removal and does not result in a change in the net calcium concentration of the blood (3).

With this exchange process in mind, it was decided to try to remove strontium from milk by means of a cation exchange resin in the calcium form.

In 1954, Nervik, Kalkstein, and Libby (4) used a cation exchange resin in a sodium form which removed both calcium and strontium from the milk. This method would necessitate replacement of the calcium content.

After the present work was started, a report by Glueckauf, Cosslet, and Watts (5) came to my attention. They employed an anion exchange resin in the chloride form to remove iodide, and suggested that radiostrontium could

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Contribution No. 7, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

be removed by passing the milk through a cation exchanger bed, which is regenerated with a mixture of calcium and sodium chloride.

Experimental

The cation exchange materials used in this study were Dowex 50W resins. They were stirred with a 20% solution of calcium chloride until the calcium concentration of the solution remained constant. The mixture was filtered through a coarse sintered glass funnel and the resin was then washed with water until the effluent was neutral and chloride-free.

The milk used in these experiments was commercial milk to which Sr^{89} was added. All tests for strontium removal were carried out by stirring the mixture of milk and resin in an Erlenmeyer flask in a variable speed shaker. The Sr^{89} content of the milk was measured by evaporating 0.2 ml milk onto a flat aluminum disk and counting in a gas flow counter.

TABLE I

Removal of Sr^{89} from milk by Ca-saturated resins

Resin	% removal
Dowex 50W-X1 (50–100)	75
Dowex 50W-X8 (50–100)	83
Dowex 50W-X12 (50–100)	84
Dowex 50W-X8 (200–400)	83
Dowex 50W-X12* (50–100)	94

NOTE: All tests were carried out on 20 ml milk with 2.0 g resin and stirred for 20 minutes. Sr^{89} was added to the milk in the amount of 2.7 μc/100 ml and 6.6 mg Sr/100 ml.

*The 20 ml of milk recovered from the first treatment with Dowex 50W-X12 was treated a second time with 2 g of the same resin for 5 minutes.

The data presented in Table I illustrate the relative efficiency of various Dowex 50W resins to remove Sr^{89} from milk. At the levels of resin employed in this experiment it would appear that porosity and particle size were of little consequence, although the high-porosity resin Dowex 50W-X1 was less efficient than the low-porosity resins. The effect of quantity of resin is shown in Table II. In this experiment Dowex 50W-X12 was used. Treatment with as little as 1.25 g of resin per 100 ml of milk removed 50% of the Sr^{89}.

TABLE II

Effect of quantity of resin and successive treatment on Sr^{89} removal

Amount resin per treatment, g	% Sr^{89} removed	
	First treatment	Second treatment
0.1	41.5	65.2
0.2	54.6	76.8
0.5	67.7	85.7
1.0	74.6	88.6

NOTE: 20 ml milk containing 3.37 μc/100 ml was stirred for 20 minutes.

An experiment designed to compare the effect of quantity of resin and successive treatment is shown in Table II. The results indicate that two shakings with

0.1 g of resin are more effective than one shaking with 0.2 g, and using 0.5 g of resin twice is more effective than one shaking with 1.0 g.

The effect of successive treatment of milk with 1.0 g of resin is shown in Table III. Three treatments removed 96.0% of the added strontium.

TABLE III

Effect on removal of strontium from milk by successive treatment with 1-g quantities of resin

No. of treatments	% removal
1	79.3
2	92.4
3	95.9
4	96.8

NOTE: Milk (20 ml volume) contained 3.37 μc Sr^{89} (9.1 mg Sr) /100 ml and was stirred for 20 minutes per treatment.

Time of shaking the resin is shown to be a relatively unimportant variable; 58.0% of the strontium is removed in the first 2.5 minutes. The results are shown in Table IV.

TABLE IV

Effect of time of stirring on removal of Sr^{89} from milk

Stirring time, minutes	% removal
2.5	58.1
5.0	64.7
10.0	65.6
20.0	69.9
40.0	70.5

NOTE: 20 ml milk containing 3.37 μc Sr^{89}/100 ml was treated with 1 g resin.

An experiment studying the effect of strontium concentration is shown in Table V. Under the conditions of this experiment the strontium concentration of the milk did not alter the percentage removed.

TABLE V

Effect of Sr concentration on removal of Sr from milk

Amount Sr per 20 ml milk, mg	% removal
0.91	76.2
1.82	77.4
2.73	74.1
3.64	75.8

NOTE: One gram of resin was used with 20 ml milk and stirred for 20 minutes.

The effect of the resin on the cation composition of the milk is shown in Table VI. In this experiment milk was stirred for 30 minutes with resin which was added to the milk in the amount of 2 g per 100 ml. The analyses indicate that treatment with a calcium-saturated resin removes some potassium and sodium and contributes some calcium.

TABLE VI

Effect of resin treatment on cation composition of milk

Resin per 20 ml milk, g	Ca, %	K, %	Na, %	Sr^{89} removed, %
0	0.116	0.162	0.051	
0.25	0.141	0.127	0.048	53.7
0.50	0.162	0.097	0.040	72.2
0.75	0.171	0.086	0.038	76.1
1.00	0.178	0.077	0.037	80.5

NOTE: Sr removal experiment was conducted with a duplicate series. The Sr content was 6.75 μc/100 ml.

A taste panel test was conducted with 20 volunteers on samples of untreated and CaKNa-treated milk. Fifty-five per cent of the volunteers felt there was a difference between the samples. Forty-five per cent could not detect any difference. Forty per cent of those that detected a difference preferred the treated milk, whereas sixty per cent preferred the untreated milk. It is concluded that there is no essential difference in flavor between the treated and untreated milk.

The change in the cation concentration of the milk brought about by treatment with a calcium-saturated resin may be considered undesirable. An attempt was therefore made to formulate another type of resin that would remove strontium, but would leave the milk unchanged.

The ratio of calcium:potassium:sodium in milk, in grams, is approximately 1:0.97:0.41. Consequently a solution was prepared containing 16.0 g $CaCl_2$, 15.5 g KCl, 6.5 g NaCl per 100 ml. Fifty grams of Dowex 50W-X12 (100–150 mesh) were stirred with five successive 200-ml portions of the above solution. After the final filtration through a coarse sintered glass funnel the cation-saturated resin was washed with water until the effluent was neutral and chloride-free. The resin was then air-dried. This resin is hereafter referred to as CaKNa resin.

The efficiency of the CaKNa resin to remove strontium from milk was tested. Twenty milliliters of milk was shaken for 20 minutes with varying quantities of the resin. The percentage of strontium removed is shown in Table VII. It appears that the CaKNa resin is at least as effective as the calcium-saturated resin.

TABLE VII

Effect of CaKNa resin treatment on Sr^{89} removal and cation composition

Amount resin per 20 ml milk, g	Ca, %	Na, %	K, %	Sr^{89} removed, %
0	0.120	0.048	0.165	
0.25	0.127	0.051	0.165	68.5
0.50	0.125	0.049	0.161	76.8
1.00	0.128	0.050	0.161	85.7

NOTE: Sr^{89} content of milk was 6.75 μc/100 ml.

The analyses of the milk before and after treatment with the CaKNa resin are shown in Table VIII. The results indicate that no significant change in

the calcium, potassium, or sodium concentration of the milk is wrought by treatment with CaKNa resin.

TABLE VIII

Effect of a resin mixture on Sr removal and cation composition

Amount resin per 20 ml milk, g	Ca, %	Na, %	K, %	Sr^{89} removed, %
0	0.120	0.048	0.165	
0.25	0.113	0.058	0.163	68.4
0.50	0.102	0.062	0.161	80.0
1.00	0.085	0.071	0.159	89.2

NOTE: Sr^{89} content was 6.75 μc/100 ml.

Another experiment was conducted with a mixture of calcium resin, potassium resin, and sodium resin. The individual resins were prepared by saturating the mixture with the chloride of the cation and then washing it with water. The resins were air-dried and mixed in the following proportions: 2.64 calcium resin:2.0 potassium resin:1.0 sodium resin.

Twenty milliliters of milk was prepared as above and treated with varying quantities of the mixed resins. The degree of strontium removal is shown in Table VIII. The analyses of the milk are shown in the same table. Strontium removal is just as efficient with the resin mixture as with the CaKNa resin. The resin mixture in the proportions employed brought about some change in the cation concentration. It is believed that the change in the composition of the milk could be avoided by altering the ratio of the different resins.

The question arises as to whether Sr^{89} which enters the milk via the physiological mechanism is as removable by the resin as is Sr^{89} which is simply added to milk. Guinea pigs which were suckling young were injected intraperitoneally with 15 μc of Sr^{89}. Three hours after injection the guinea pig was milked by mild suction. Approximately 0.5 ml of the guinea pig milk containing Sr^{89} was diluted to 10 ml with commercial cow's milk. The resulting milk solution was treated with CaKNa resin, 0.25 g resin per 10 ml milk, for 20 minutes. After the first shaking the milk was filtered off, sampled, and treated again in the same manner. The first treatment removed 75.7% of the Sr^{89} and the combined first and second treatments removed 90.2%. This result indicated that Sr^{89} which enters milk via the milk secretion mechanism is removed just as efficiently as artificially "strontified" milk.

TABLE IX

Effect of quantity of resin and successive treatment on Cs^{137} removal

Amount resin per treatment, g	% Cs^{137} removed	
	First treatment	Second treatment
0.1	49.2	73.0
0.2	56.6	82.4
0.5	67.6	87.1
1.0	75.7	92.1

An experiment was conducted with milk to which Cs^{137} was added in the amount of 1 μc/100 ml. The results of the cesium removal are shown in Table IX. The Cs^{137} was counted in the gas flow counter. The results indicate that cesium can be removed in the same manner as strontium. The cesium-containing milk was treated successively with different quantities of resin. The results shown in Table IX indicate that two treatments with 1 g resin per 20 ml milk resulted in approximately 92% removal.

Conclusions

Cation exchange resin in a calcium form is able to remove strontium from milk. This treatment alters the cation concentration of the milk. Resin that is equilibrated with a solution of calcium, potassium, and sodium chlorides, in which the ratio of these cations is the same as that which exists in milk, is just as efficient with respect to strontium removal, and also removes cesium. This resin does not alter the cation composition of the milk.

Approximately 70% of the strontium can be removed from 1 liter of milk with 25 g of resin by simple batch treatment. More efficient removal is obtainable with larger quantities of resin. Several treatments with a given quantity of resin are more efficient than a single treatment with the same quantity.

Neither the concentration of strontium in milk, nor the source of strontium, has any effect on the efficiency of removal. The indications are that removal of strontium and cesium from milk is feasible. The question remains whether the process can be placed on a commercial basis if it ever becomes necessary. The answer awaits further investigation.

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EVALUATION OF PROTEIN IN FOODS

IV. A SIMPLIFIED CHEMICAL SCORE[1]

J. M. McLAUGHLAN, C. G. ROGERS, D. G. CHAPMAN, AND J. A. CAMPBELL

Abstract

Available evidence has shown that most common foods are deficient in lysine, methionine, or in methionine and cystine. Based on the determination of these amino acids, a simplified chemical score was developed and compared with protein efficiency ratio (P.E.R.) values determined with the same samples. Each of 43 foods was assigned to either of two categories: (*a*) foods apparently deficient in lysine or (*b*) in methionine (+ cystine). With 16 foods in the former group there was a high degree of correlation between lysine concentration and P.E.R. With 27 foods in the latter group, a good correlation was found between the methionine (+ cystine) concentration and P.E.R. Since the regression lines for the two groups were different, a factor was added to the methionine (+ cystine) values to simplify the relationship. That foods deficient in lysine or in methionine (+ cystine) fell into two distinct groups appeared to be substantiated by data available in the literature. Because the simplified method is relatively rapid, yields reproducible results, and correlates with animal assays, it is proposed as a rapid screening procedure for the evaluation of protein in foods, but is not intended to replace the rat bio-assay method.

Introduction

The advantages of a simple screening procedure for protein evaluation are well recognized. Several workers have suggested the use of bacteria or protozoa in rapid screening tests, but these methods have been subject to some criticism (1). Furthermore, previous work in this laboratory (2) showed that bacteriological methods gave erroneous results with many foods when compared with a standardized P.E.R. assay (3). They did not allow for the methionine–cystine sparing relationships observed with rats and, consequently, were found to be unsuitable for legumes and animal proteins.

Block and Mitchell (4) showed that a close relationship existed between chemical score and nutritive value of proteins as judged by biological value or protein efficiency ratio determinations. The chemical score was based on the estimate of the amino acid in maximum deficit relative to whole egg protein after first establishing the relative concentrations of 10 amino acids in the proteins. For cereal products the chemical score was usually based upon the lysine concentration.

Recently the Food and Agricultural Organization (5) proposed that proteins be rated by comparison with a provisional mixture of pure amino acids, based on the requirements of adult humans. This method placed less emphasis than chemical score upon lysine, but more emphasis upon tryptophan and consequently tryptophan rather than lysine was considered to be the most deficient amino acid in some cereals. Evidence is available to show, however, that the nutritive value of cereals and cereal products may be predicted from their lysine

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content (2, 6). Furthermore, it has been suggested (7, 8) that in most practical foods the amino acids limiting growth are lysine or methionine (+ cystine) or, possibly, tryptophan.

In this paper it is shown that a simplified chemical score, based upon the lysine, methionine, and cystine content of foods, correlates well with results of P.E.R. determinations on the same foodstuffs, and hence, may be useful as a simple screening procedure for the evaluation of protein in foods.

Methods

Principle

Lysine, methionine, and cystine were estimated by microbiological assay in 2-hour acid hydrolyzates of food proteins. The values obtained were expressed as a percentage of the concentration of each amino acid in hydrolyzed egg protein. A simplified chemical score (S.C.S.) for a food was calculated as in the original method (4) (i.e. where cystine was the most deficient amino acid, the combination of methionine and cystine was used in calculating the score; when methionine was the most deficient amino acid, only the methionine content was used in calculating the S.C.S.).

Determination of Amino Acids

The procedure for acid hydrolysis of proteins and methods of assay for lysine and cystine have been described (2). *Leuconostoc mesenteroides* P-60 A.T.C.C. No. 8042 was used for the assay of methionine in 2-hour acid hydrolyzates by the method of Steele *et al.* (9) employing Difco* methionine assay medium.

Determination of Protein Efficiency Ratio

The method of determination of protein efficiency ratio was that previously described by Chapman, Castillo, and Campbell (3).

Results

Amino acid values, S.C.S., and P.E.R. for each of 16 protein foods apparently deficient in lysine are given in Table I. Each amino acid value was expressed as a percentage of the concentration in egg which was arbitrarily set at 100. Similar data for each of 27 protein foods apparently deficient in methionine or methionine and cystine are shown in Table II.

The correlation between S.C.S. and P.E.R. for food proteins apparently deficient in lysine and in methionine (+ cystine) is shown in Fig. 1. It can be seen from the calculated regression lines that the proteins fall into two distinct groups. A high degree of correlation ($r = +0.96$) was found between S.C.S. and P.E.R. for protein foods deficient in lysine. A good correlation ($r = +0.83$) was also obtained between S.C.S. and P.E.R. for protein foods deficient in methionine (+ cystine). The difference between the two curves at the mean of the methionine (+ cystine) score (56.25) was approximately 15 units of S.C.S. To permit the use of a single-scoring system the methionine (+ cystine) values were adjusted by adding a factor of 15. After adjustment, the S.C.S. values for

*Difco Laboratories, Detroit, Michigan, U. S. A.

TABLE I

Amino acid values, S.C.S.,* and P.E.R.† for food proteins apparently deficient in lysine

Source of protein	% of egg				Simplified chemical score	Protein efficiency ratio
	Lysine	Methionine	Cystine	Methionine + cystine		
Whole egg	100	100	100	100	100	3.35
"Protein Cereal" A	68	85	43	68	68	2.04
Mixed Baby Cereal	67	68	66	67	67	2.18
"Protein Cereal" B + milk	63	70	58	65	63	2.19
Oats + wheat gluten	48	60	145	94	48	1.75
Wheat germ cereal	39	45	76	57	39	1.59
"Protein Cereal" B	20	51	49	50	20	0.03
"Protein" bread	44	45	72	56	44	1.29
Roman meal bread	37	47	75	58	37	1.16
White bread	35	56	80	66	35	0.77
Whole wheat bread	31	46	61	52	31	1.09
Wheat gluten bread	31	57	77	65	31	0.90
Wheat gluten rolls	18	60	87	71	18	0.13
Whole wheat flour	31	38	82	56	31	1.17
White flour	26	46	74	57	26	0.59
Wheat gluten flour	23	—	—	—	23	0.52

*S.C.S. is an abbreviation for simplified chemical score.
†P.E.R. is an abbreviation for protein efficiency ratio.

foods deficient in methionine (+ cystine) were combined with those for foods apparently deficient in lysine and the data replotted as shown in Fig. 2. The combined data for all foods correlated closely ($r = +0.93$) with their respective P.E.R. values.

These observations suggested that a similar approach could be applied to the data of Block and Mitchell (4). It was found that many protein foods used in their study could also be divided into 2 groups, one deficient in lysine and the other in methionine (+ cystine). Where sufficient data were given, S.C.S. values were calculated and compared with the corresponding P.E.R. estimates and chemical score values. The results are summarized in Table III. The values shown for S.C.S. are identical with the chemical scores calculated by Block and Mitchell since both are based on values for lysine or methionine (+ cystine). The correlation between S.C.S. and P.E.R. for these foods is shown in Fig. 3. Although relatively few values are included, the data fell, apparently, into two groups in a manner similar to that obtained with the data shown in Fig. 1.

Of 43 foods listed in Block and Mitchell's report, 37 were apparently deficient in either lysine or methionine (+ cystine). For these foods the S.C.S. and chemical score values were identical. Those foods that were indicated to be deficient in some other amino acid are listed in Table IV, along with their S.C.S. and chemical score values. The two scores were almost identical for three foods and similar for the remainder.

TABLE II

Amino acid values, S.C.S.,* and P.E.R.* for food proteins apparently deficient in methionine + cystine

Source of protein	% of egg				Simplified chemical score	Protein efficiency ratio
	Lysine	Methionine	Cystine	Methionine + cystine		
Fish flour	160	116	41	86	86	3.04
Casein	136	107	13	89	69	2.50
Cheddar cheese	133	95	27	68	68	2.32
Hamburger (dried)	114	89	32	66	66	2.68
Whole milk powder	116	89	29	65	65	2.56
Oats + wheat gluten + milk	79	73	78	75	73	2.69
"Protein Cereal" C	85	72	69	71	71	2.66
Oatmeal + milk	78	70	70	70	70	2.90
Corn Flakes + milk	78	89	42	70	70	2.64
"Protein Cereal" D	84	74	60	68	68	2.66
Puffed Oat cereal + milk	82	74	47	63	63	2.56
Soybean cereal + milk	99	68	50	61	61	2.62
Shredded Wheat + milk	79	70	43	59	59	2.65
Puffed Corn cereal + milk	80	76	30	58	58	2.90
Bran cereal + milk	80	69	40	57	57	2.15
Soybean cereal	87	50	56	53	50	2.42
"Protein Cereal" F	70	47	67	55	47	2.21
"Protein Cereal" E	69	46	48	47	46	2.39
"Protein Cereal" G	55	44	95	65	44	2.16
Oatmeal	53	44	130	79	44	2.13
Puffed Wheat cereal + milk	47	51	22	40	40	1.90
Wheat germ (bulk)	99	57	43	51	51	2.52
Wheat germ, cereal supplement	93	56	41	50	50	2.57
Lima beans (cooked)	115	54	34	46	46	1.72
Soybean flour	78	42	42	42	42	2.04
White wax beans (cooked)	102	42	28	36	36	1.59
Peas (cooked)	110	32	47	38	32	1.57

*See footnotes in Table I.

TABLE III

S.C.S.* and P.E.R.* for foods apparently deficient in lysine† and in methionine + cystine

Limiting in lysine			Limiting in methionine + cystine		
Source of protein	Simplified chemical score	Protein efficiency ratio	Source of protein	Simplified chemical score	Protein efficiency ratio
Whole egg	100	3.8	Beef muscle	71	3.2
Egg albumin	69	2.6	Cow's milk	68	2.8
Rolled oats	46	2.2	Lactalbumin	66	2.9
White rice	44	1.7	Beef kidney	65	2.9
Whole wheat	37	1.5	Casein	58	2.0
Cottonseed meal	37	1.3	Soybeans	49	2.3
Whole maize	28	1.2	Maize germ	39	2.6
White flour	28	1.0	Peanuts	24	1.7
Maize gluten	11	0.7	Peas	24	1.1

*See footnotes in Table I.
†Data taken from Block and Mitchell (4).

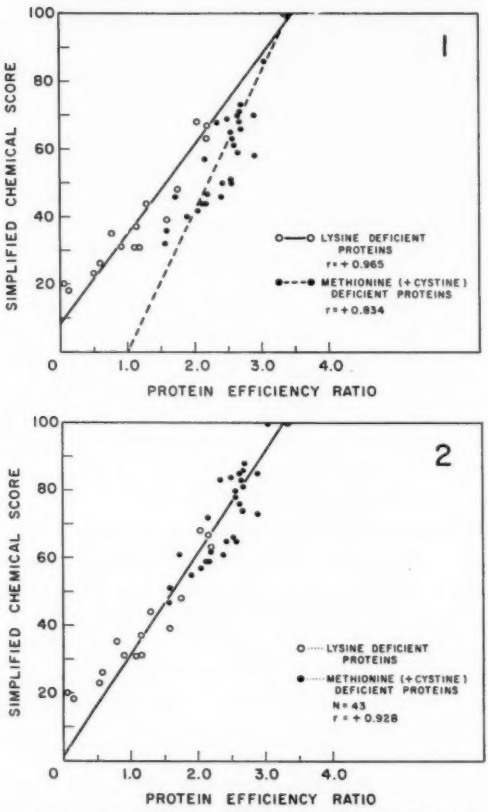

FIG. 1. Correlation between S.C.S. and P.E.R. for 43 foods. Foods apparently deficient in lysine are plotted separately from those apparently deficient in methionine (+ cystine).
FIG. 2. Correlation between S.C.S. and P.E.R. for 43 foods after adjustment of the (methionine + cystine) scores.

TABLE IV

Foods with different ratings by S.C.S.* and chemical score†

Food	Chemical score		S.C.S.	
	"Limiting"‡ amino acid	Score	"Limiting"‡ amino acid	Score
Egg albumin	Thre.	78	Lys.	90
Liver	Isol.	70	Meth. cyst.	71
Heart	Isol.	65	Meth. cyst.	68
Brain	Isol.	64	Meth. cyst.	74
Wheat germ	Isol.	38	Meth. cyst.	42
Gelatin	Tryp.	0	Meth. cyst.	14

*See footnote in Table I.
†Data taken from Block and Mitchell (4).
‡Not necessarily the most limiting amino acid for growth of animals.

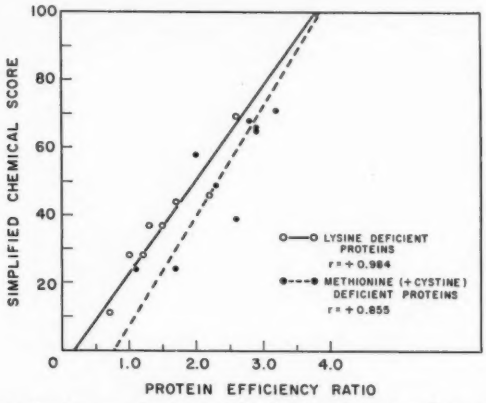

FIG. 3. Correlation between S.C.S. and P.E.R. for 18 foods. The amino acid analyses and P.E.R.'s were taken from Block and Mitchell's data (Nutrition Abstr. & Rev. **16**, 249 (1946).

Discussion

The foregoing observations have shown that the determination of three amino acids, lysine, methionine, and cystine, is sufficient for the estimation of the nutritive value of the protein of common foods. Examination of the data of Block and Mitchell indicated that the proposed method yields essentially the same results as chemical score (using 10 amino acids) even for foods in which the most deficient amino acid may be isoleucine, or tryptophan. If future work should show that isoleucine, or some other amino acid, is most deficient in a significant number of practical foods, then the proposed method could be modified to include that amino acid.

The agreement with P.E.R. for foods deficient in methionine or in methionine (+ cystine) was not as good as that for foods deficient in lysine and this observation may be related to the assumption that methionine and cystine are equivalent when computing the score. When the methionine–cystine sparing relationship is understood more fully a more accurate score may be calculated and, presumably, a higher correlation might then be obtained between methionine (+ cystine) content and P.E.R.

Many of the foods examined were complex mixtures of vegetable proteins, such as "protein" breakfast cereals, several of which were tested with and without milk. This suggested that the simplified chemical score may be useful in predicting the relative nutritive value of complete mixed diets.

The observation that foods deficient in lysine or in methionine (+ cystine) fell into two groups when compared with their appropriate P.E.R.'s suggested that relative to the rats' requirement, whole egg had a surplus of methionine (+ cystine); this observation is in agreement with the work of Flodin (10). Since the correction factor is dependent upon the reliability of the amino acid assays and hence is subject to bias it would be preferable for individual laboratories to establish their own factors. This could be done by relating amino acid values, as determined in their laboratories, to accepted P.E.R. values for several

selected foodstuffs. Instead of rating foods on the basis of S.C.S., however, P.E.R.'s could be predicted directly from the two "established" regression lines.

It was found convenient to use the 2-hour acid hydrolysis procedure of Horn and Blum (11) for the assay of lysine and methionine. A considerable saving of time was achieved by the use of one hydrolyzate for the assay of the three amino acids. Expressed in terms of milligrams of amino acid per gram of protein, lysine and methionine were approximately 80% available, microbiologically, after a 2-hour acid hydrolysis. When amino acids were expressed as a percentage of the concentration in egg, similar results were obtained with either 2- or 8-hour hydrolyzates.

Although the proposed method gives less information than chemical score concerning the order of amino acid deficiencies in a food, it was found to give a reliable estimate of protein quality for the foods examined. Like chemical score or the Food and Agriculture Organization method, simplified chemical score does not take into account variation in digestibility or possible heat damage. The proposed method is not intended to replace animal or human bio-assay procedures as the principal criteria of protein quality. However, provided that its limitations are kept in mind, the method should be useful as a relatively simple, and rapid screening, procedure for predicting the nutritive value of the protein in foods.

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OXIME REACTIVATION STUDIES OF INHIBITED TRUE AND PSEUDO CHOLINESTERASE[1]

J. F. SCAIFE

Abstract

Electric eel cholinesterase and purified pseudo cholinesterase from horse serum were inhibited with sarin, tabun, or O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate. The inhibited enzymes were then reactivated at 25° C with one of the oximes: pyridine 2-aldoxime methiodide, 1,1′-trimethylene bis(4-hydroximinomethyl pyridinum) bromide, and 1-(3-triethylammoniopropyl)-4-hydroximinomethyl pyridinium dibromide. Two kinetically different types of reactivation were found; in one, reactivation was progressive with time, while in the other it was rapid initially followed by an equilibrium condition which was dependent upon the concentration of uninhibited enzyme in the system. The type of reactivation varied according to the nature of the enzyme, oxime, or inhibitor under investigation. Reactivation was always found to be dependent upon the concentration of oxime and inhibited enzyme, pH, and the presence of substrate. The rate of change at 25° C of the inhibited enzymes to a form incapable of being reactivated has been assessed.

Introduction

Cholinesterase inhibited by organophosphorus compounds can be reactivated with variable success by a wide variety of oximes (1–6). The kinetics of reactivation have been found to vary with the reactivator and its concentration. They may be zero order (7), first order (8), or bimolecular (9). In the case of quaternary compounds a preliminary complex is apparently formed between the inhibited enzyme and the oxime (10–12). It has been noted that the inhibited enzyme can change progressively into a form incapable of being reactivated (1, 2, 8) and consequently reactivation studies must be made on freshly inhibited enzyme prepared in the cold.

Residual traces of excess inhibitor must be eliminated from the preparation prior to studies on reactivation because of the extreme sensitivity of the enzyme to most of the inhibitors. If red cells are used as the source of the enzyme, this can be accomplished by repeated washings (11, 12). Crude enzyme preparations such as brain, liver, or serum often contain enzymes which can destroy traces of excess inhibitors (13–16). Dilution has been used to reduce the concentration of residual inhibitors to insignificant levels in purified preparations of high activity (4). Further dilution after the reactivation step has also been used as a means of stopping the reactivation process, and allowing time for the measurement of the reactivated cholinesterase. The present work has demonstrated that such a step is undesirable, as it may disrupt the equilibrium type of reactivation found in certain cases and leads to fallacious results. For our investigation purified samples of electric eel cholinesterase and of horse serum cholinesterase were used to study reactivation.

The recent development of the powerful cholinesterase reactivators, 1,1′-trimethylene bis(4-hydroximinomethyl pyridinium) bromide (I) (17–19) and

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1-(3-triethylammoniopropyl)-4-hydroximinomethyl pyridinium dibromide (II) has made it possible to effectively extend previous reactivation studies to include pseudo cholinesterase, which is more difficult than cholinesterase to reactivate, and cholinesterase inhibited with tabun, which up to the present time has not been effectively reactivated by other oximes.

Methods and Materials

Sarin (isopropyl methylphosphonofluoridate), tabun (ethyl N,N-dimethylphosphoramidocyanidate), and O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate (DSDP), were prepared in this laboratory and were at least 95% pure, as determined from infrared spectra. Solutions were made up in water immediately prior to use.

The sample of electric eel cholinesterase, kindly supplied by Dr. B. Jandorf of the U.S. Army Chemical Centre, Md., had a hydrolytic activity of 40.7 g acetylcholine per hr per ml at 25° C. A sample of horse serum pseudo cholinesterase, obtained from the Rijksverdedigingsorganisatie, T.N.O., Utrecht, Netherlands, had an activity of 0.113 g benzoylcholine per hr per mg at 25° C.

Pyridine 2-aldoxime methiodide (2-PAM) was prepared in these laboratories and had a melting point of 234° C. Oxime I, 1,1′-trimethylene bis(4-hydroximinomethyl pyridinium) bromide, and oxime II, 1-(3-triethylammoniopropyl)-4-hydroximinomethyl pyridinium dibromide, were obtained from the U. S. Army Chemical Centre, Md.

Veronal buffer (0.02 *M*) of pH 7.4 contained 0.3 *M* potassium chloride and 0.1% bovine plasma albumin.

Method for Producing Inhibited Enzymes

(*a*) *Eel cholinesterase.*—Five microliters of stock enzyme solution (above) was added to 0.2 ml of veronal buffer and then 5 μl of inhibitor solution containing 0.01 mm^3 per ml and the mixture was left overnight at +1° C. It was then diluted to 5 ml with cold 0.1% bovine plasma albumin. If the inhibitor used was sarin or DSDP the solution was extracted 4 to 5 times with equal volumes of cold, peroxide-free diethyl ether. The excess dissolved ether was then removed from the solution by a jet of air impinging on the surface. This treatment had no effect upon controls containing the uninhibited enzymes. Solutions containing tabun were dialyzed against several changes of ice-cold water.

(*b*) *Pseudo cholinesterase.*—A weighed quantity of approximately 1–2 mg of the enzyme preparation was dissolved in 0.2 ml of veronal buffer and 5 μl of inhibitor solution was added as above. The mixture was left overnight at +1° C and then was diluted with 0.1% bovine plasma albumin to contain 1.25 mg of the enzyme preparation per 5 ml and then treated as above.

The inhibited enzyme solutions were considered to be free of excess inhibitor when equal volumes of inhibited and uninhibited enzyme were incubated together for 30 minutes in a Warburg vessel at 25° C and no change in the activity of the uninhibited enzyme was demonstrated.

Reactivation and Measurement of Cholinesterase Activity

Reactivations were carried out at 25° C in double-arm Warburg vessels. One side arm contained the oxime solution; the other the substrate. The enzyme preparation and all other materials were contained in the main compartment. Except for the determinations made over a range of pH values, all measurements of reactivation were conducted at pH 7.43 under the following conditions: 1.5 ml of 0.05 *M* sodium bicarbonate, 0.5 ml of 0.6 *M* sodium chloride, 0.1 ml of 0.6 *M* magnesium chloride, 0.1 ml of 3.0% bovine plasma albumin, 0.1–0.2 ml of oxime solution, and inhibited enzyme (0.1 ml of eel cholinesterase and 0.2 ml of pseudo cholinesterase). The volume of solution was adjusted to 2.9 ml with water. The gas phase used for equilibration was 5% carbon dioxide in nitrogen. After equilibration, the oxime solution, previously adjusted to the final pH of the mixture with 0.1 *N* sodium hydroxide, was added to the main vessel. Following the period required for reactivation, 0.1 ml of the substrate solution was added from the other side arm. For eel cholinesterase the substrate was 0.3 *M* acetylcholine bromide, while for pseudo cholinesterase 0.45 *M* benzoylcholine chloride was used.

Control vessels without oximes were run at the same time for the evaluation of the activity of the uninhibited and inhibited enzyme preparations alone. The rate of the non-enzymatic hydrolysis of substrate was also determined. The degree of inhibition produced by the oximes themselves was also determined for several concentrations of the oxime.

Reactivations at different pH values were carried out in sodium bicarbonate – carbon dioxide buffers. Solutions were equilibrated with either 100% or 5% carbon dioxide in nitrogen and the concentration of bicarbonate was varied so that the pH range covered was from 5.73 to 8.34. The 0.2 *M* final concentration of sodium ions was maintained by the addition of varying amounts of sodium chloride. The oxime solutions were adjusted to the appropriate final pH of the solutions in each vessel, before addition from the side arm. Control determinations of the activity of the uninhibited and inhibited enzymes were made at each pH value.

The rate at which the inhibited enzymes are converted into nonreactivatable forms has been determined at 25° C for each inhibitor. Equal volumes (0.4 ml) of inhibited enzyme and 0.02 *M* veronal buffer of pH 7.4, were mixed and incubated in stoppered test tubes at 25° C for varying periods of time, after which an aliquot was reactivated as described above. From these results a correction was applied to the experimentally determined reactivations to compensate for the amount of irreversible change, which had occurred at 25° C to the inhibited enzyme, before the addition of the oxime. In these studies the amount of reactivatable enzyme was never found to be less than 98% of the uninhibited enzyme originally present.

Calculation of the Per Cent Reactivation

After the desired period of time for reactivation of the inhibited enzyme by an oxime, the cholinesterase activity of the solution was measured manometrically. It was thus possible to express cholinesterase activity in the reactivated

solutions in terms of the initial velocity of evolution of carbon dioxide in microliters per minute and compare it with the reaction velocity for the uninhibited enzyme preparation. Under certain circumstances, when the time of reactivation was short, or the concentration of oxime was low, the activity of cholinesterase in the reactivated solutions was not constant, but increased progressively with time after the addition of the substrate. In these cases there was some small degree of uncertainty in estimating the initial velocity of the reaction.

The calculation of the per cent reactivation has been accomplished using the following formula:

$$\text{Per cent reactivation} = \frac{V_r - V_i}{V_0 f.c - V_i} \cdot 100$$

where:

V_r = initial reaction velocity of the reactivated mixture, after subtraction of the velocity of the non-enzymatic (blank) reaction, in μl per min.

V_0 = initial reaction velocity of the uninhibited enzyme solution, after subtraction of the blank, in μl per min.

V_i = initial reaction velocity of the inhibited enzyme solution, after subtraction of the blank, in μl per min. This term is usually zero.

f = factor for reducing the activity of the uninhibited enzyme due to the inhibition of the oxime, at the concentration used for reactivation.

c = fraction of the enzyme still reactivatable prior to the addition of the oxime.

Results

Oxime Concentration

The effect of oxime concentration on the reactivation of eel cholinesterase inhibited with DSDP has been set out in Table I. The results for eel cholinest-

TABLE I

Reactivation of eel cholinesterase inhibited with DSDP (reactivation time 30 minutes)

Oxime concentration ($M \times 10^{-4}$	Reactivation, %		
	Oxime I	2-PAM	Oxime II
13.8	98	100	100
6.9	98	98	98
0.69	91	90	90
0.138	86	84	77
0.069	83	81	70
0.0345	80	78	55
0.0138	73	71	34
0.0069	68	64	—
0.00345	60	—	—
0.00173	46	—	—

erase inhibited with sarin or tabun are shown in Figs. 1 and 2. In all cases increasing the concentration of oxime results in an increased degree of reactivation. In the case of tabun, however, reactivation with oxime II even at the highest concentrations used has resulted in only partial reactivation. The

shapes of the concentration – per cent reactivation curves are also different for sarin- and tabun-inhibited eel cholinesterase.

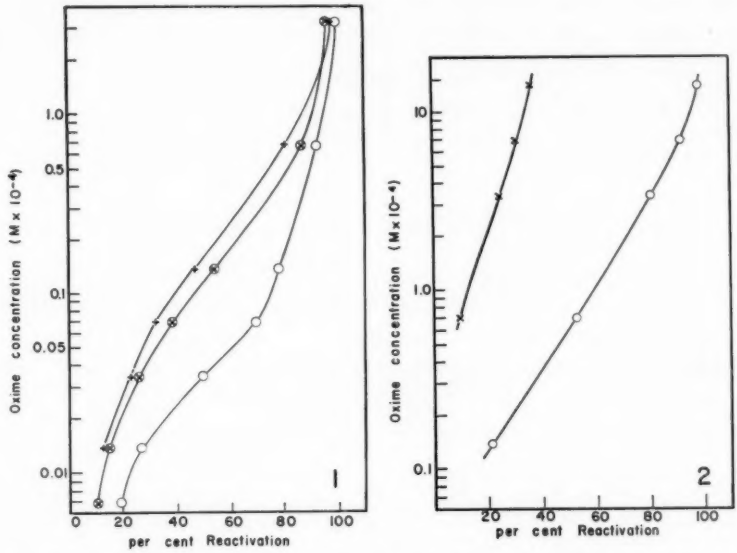

FIG. 1. Reactivation of eel cholinesterase inhibited with sarin. Oxime I, ○; 2-PAM, ⊕; oxime II, +. Reactivation time, 15 minutes.
FIG. 2. Reactivation of eel cholinesterase inhibited with tabun. Oxime I, ○; oxime II, ×. Reactivation time, 30 minutes.

TABLE II

Reactivation of pseudo cholinesterase inhibited with sarin, tabun, or DSDP

	Oxime concentration ($M \times 10^{-4}$)	Reactivation in 30 min, %		
		Oxime I	2-PAM	Oxime II
Sarin	34.5	52	48	46
	13.8	50	46	44
	6.9	46	43	42
	0.69	29	24	12
Tabun	34.5	27	5	8
	13.8	12	2	4
DSDP	34.5	66	37	52
	13.8	57	32	28
	6.9	45	25	13
	0.69	8	5	2

The results obtained using pseudo cholinesterase are given in Table II. It will be seen that, comparing the same inhibitors, much higher concentrations of oxime are required to effect a degree of reactivation equivalent to that obtainable with eel cholinesterase.

Time

To study the effect of time on the reactivation, inhibited cholinesterase was treated with a constant concentration of oxime for varying times. The results for eel cholinesterase inhibited with tabun are set out in Table III and for pseudo cholinesterase in Table IV. The effect of time on the reactivation is

TABLE III

Effect of time of reactivation on eel cholinesterase inhibited with tabun

Oxime	Concentration ($M \times 10^{-4}$)	Time (min) 5	15	30	60
		Reactivation, %			
I	6.9	80	83	92	96
II	6.9	14	20	31	43

TABLE IV

Effect of time of reactivation on pseudo cholinesterase inhibited with sarin, tabun, or DSDP

Oxime	Concentration ($M \times 10^{-3}$)	Inhibitor	Time (min) 5	15	30	60
			Reactivation, %			
I	1.38	DSDP	53	55	57	59
	3.45	Sarin	—	52	52	52
	0.069	Sarin	18	28	29	29
II	1.38	DSDP	17	23	28	35
	3.45	Sarin	—	21	46	46
	0.69	Sarin	—	42	42	42
2-PAM	1.38	DSDP	18	26	32	41
	3.45	Sarin	—	43	48	50
	0.069	Sarin	—	15	24	25

also shown graphically in Figs. 3 and 4. Two types of reactivation can be distinguished for both enzymes. In one case there is an initial rapid reactivation after which little or no further change occurs. In the other case, reactivation proceeds progressively with time but becomes slower as the reaction progresses. It will be seen that the type of reactivation is dependent not only on the oxime and the inhibitor group attached to the enzyme, but also on the enzyme itself.

Substrate

The effect of substrate upon the progress of the reactivation of eel cholinesterase inhibited with sarin and reactivated by 2-PAM is shown in Fig. 5. The addition of substrate to the reactivation mixture along with oxime retards the rate of reactivation, especially at lower concentrations of oximes. This effect has been noticed with all the oximes examined with both eel cholinesterase and pseudo cholinesterase.

The results obtained for the reactivation of eel cholinesterase and pseudo cholinesterase after incubation at 25° C in veronal buffer of pH 7.4 are set

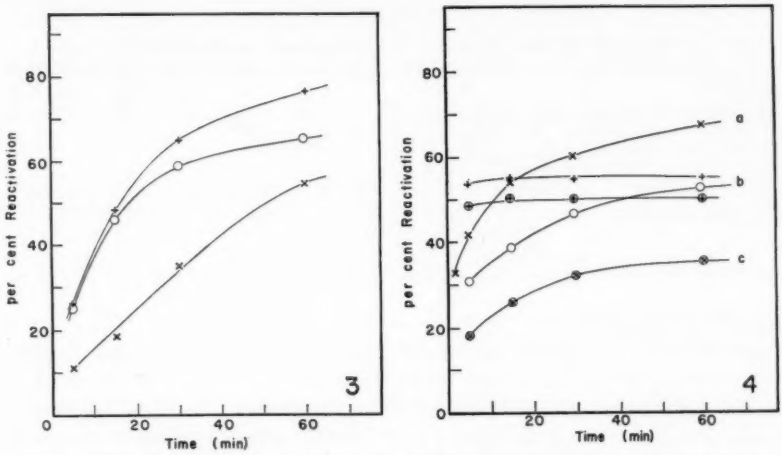

FIG. 3. Reactivation of eel cholinesterase inhibited with DSDP. Oxime I, 0.345×10^{-6} *M*, ○; 2-PAM, 0.690×10^{-6} *M*, +; oxime II, 1.38×10^{-6} *M*, ×.

FIG. 4. Reactivation of eel cholinesterase inhibited with sarin. Oxime I, 3.45×10^{-6} *M*, ⊕; 2-PAM, 13.8×10^{-6} *M*, *a*, 6.9×10^{-6} *M*, *b*, 3.45×10^{-6} *M*, *c*; oxime II, 20.0×10^{-6} *M*, +.

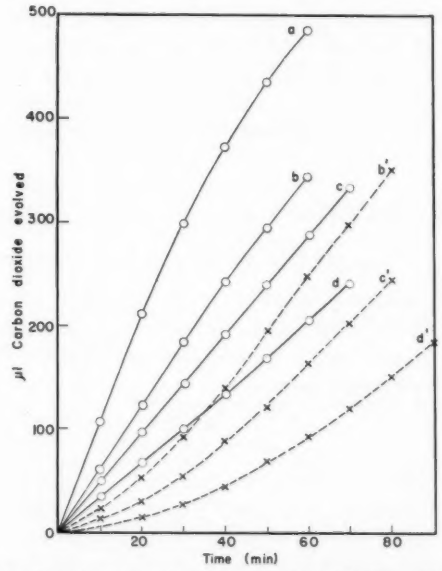

FIG. 5. Effect of substrate on reactivation. ○ Acetylcholine added after 30 minutes' reactivation. × Acetylcholine added simultaneously with the oxime. Concentration of 2-PAM *a*, 0; *b*, 13.8×10^{-6} *M*; *c*, 6.9×10^{-6} *M*; *d*, 3.45×10^{-6} *M*.

out in Table V. These figures were used to correct the experimentally determined reactivation results for the irreversible reactivation changes occurring to the enzyme before the addition of the oxime.

TABLE V

Rate of change of cholinesterase to a non-reactivatable form, at 25° C and pH 7.4

Time (hr)	0	2	5	10	16	22
% change, sarin-inhibited eel cholinesterase	0	6	14	26	37	47
Rate of change (% per hr)						
Eel cholinesterase with sarin	2.8					
Tabun	1.6					
DSDP	0.95					
Pseudo cholinesterase with sarin	0.31					
Tabun	—					
DSDP	0.95					

Enzyme Concentration

The effect of the concentration of inhibited enzyme upon the degree of reactivation has been investigated in two ways, for the case of eel cholinesterase inhibited with sarin and reactivated with oxime I. First, the concentration of inhibited enzyme was varied fourfold, and the per cent reactivation by a constant concentration of oxime determined. Second, mixtures of inhibited and uninhibited enzyme were made in varying proportions, and the per cent reactivation of a constant total amount of each enzyme mixture determined in the same manner as above. It was found that in the first case, the per cent reactivation decreased with increasing concentration of the inhibited enzyme. This was also true for sarin-inhibited eel cholinesterase reactivated with 2-PAM. In the second case, the presence of increasing proportions of uninhibited enzyme in the mixtures reduced the degree of reactivation of the inhibited enzyme fraction. These results are set out in Table VI.

TABLE VI

Effect of enzyme concentration on the reactivation of sarin-inhibited eel cholinesterase with oxime I

Inhibited enzyme alone				
Enzyme added per vessel (ml)	0.2		0.1	0.05
Calculated concentration ($M \times 10^{-8}$)	5.76		2.88	1.44
% reactivation	39		50	62
K'	0.0042		0.0042	0.0042
Inhibited and uninhibited enzyme mixture				
% inhibited enzyme in 0.1 ml total volume	90	75	50	25
% reactivation	48	45	41	35
K'	0.0042	0.0042	0.0042	0.0038

Influence of pH

The effect of pH upon the reactivation of cholinesterase inhibited with sarin and tabun is demonstrated in Table VII. In these experiments the concentration of sodium ions was maintained constant at a final value of 0.2 M, since previous workers (20–22) have shown that the optimum pH for reactivation is altered by the presence of salts. It should be mentioned that attempts to measure the effect of pH on the reactivation by the dilution technique (4) did not demonstrate such marked effects of pH on the reactivation as were found using the above method.

TABLE VII

Effect of pH on the reactivation of inhibited eel cholinesterase

	pH						
	5.73	6.42	7.03	7.43	7.72	8.02	8.34
	Reactivation, %						
Sarin reacted with 2-PAM (0.069×10^{-4} *M*)	5	13	34	47	46	43	39
Tabun reacted with oxime I (3.45×10^{-4} *M*)	8	32	63	81	80	73	67

Discussion

The results demonstrate that as far as the reactivation of cholinesterase is concerned, oxime I is superior to either 2-PAM or oxime II, for both true and pseudo cholinesterase. The differences between the reactivating powers of the oximes is, however, very much dependent upon the type of inhibitor and the enzyme under consideration. Moreover, a very important consideration, the time of reactivation must be taken into account in making comparisons, since these findings clearly demonstrate two different types of reactivation. In one case, the reactivation is time-dependent and resembles a normal second order reaction. This type of reactivation has been that most frequently reported by workers until the present time, although an examination of the results of other workers (4) reveals an apparent equilibrium condition in the reactivation of TEPP-inhibited eel cholinesterase by 2-PAM. The second type of reactivation is that exemplified by oximes I and II on sarin-inhibited eel and pseudo cholinesterase. After an initial rapid reactivation, little further change occurs in the degree of reactivation of the inhibited enzyme. Apparently an equilibrium condition is attained.

A simple expression of such an equilibrium for the reactivation would be as follows:

[1] $$\text{EI} + \text{oxime} \rightleftarrows \text{E} + \text{I. oxime}$$

The stability of the phosphorylated oxime reactivation product, I. oxime, would thus determine whether further reactivation would result after the attainment of equilibrium. The equilibrium constant would thus be expressed as:

[2] $$K' = \frac{[\text{E}]\ [\text{I. oxime}]}{[\text{EI}]\ [\text{oxime}]}.$$

If the initial concentration of inhibited enzyme is a, and the amount of free enzyme formed by reactivation with a concentration of oxime b, is x, then

[3] $$K' = \frac{x^2}{(a-x)\ (b-x)}.$$

If the fraction of the inhibited enzyme reactivated is r, then $r = x/a$ so that

[4] $$K' = \frac{r^2}{\{(b/a)-r\}\ (1-r)}$$

and K' can be evaluated provided that a is known. A value can be assigned to a by assuming a turnover number of 20×10^6 for eel cholinesterase (23).

In the case where mixtures of inhibited and uninhibited enzyme are reactivated by a constant concentration of oxime, K' can be similarly evaluated. If the amount of uninhibited enzyme initially present in the mixture is E, then

$$[5] \qquad K' = \frac{(\mathrm{E}+x)\ (x)}{(a-x)\ (b-x)} = \frac{\mathrm{E}+ar}{\{(1/r)-1\}\ (b-ar)}.$$

Thus in the case of inhibited enzyme alone, equation [4] shows that a decrease in the concentration of the inhibited enzyme should increase the per cent reactivation. For mixtures containing uninhibited enzyme, equation [5] reveals that the per cent reactivation of the inhibited fraction should decrease as the amount of uninhibited enzyme in the mixture increases. Both of these predictions were substantiated in fact, from the experimental results as set out in Table VI. The calculation of K', however, at a constant enzyme concentration but over a range of oxime concentrations, did not give a constant value. Thus from the results of Fig. 1 with oxime I over the range of concentration 1.38×10^{-6} M to 13.8×10^{-6} M K' varies from 0.0031 to 0.0062. Thus the equilibrium condition expressed by equation [1] must be in reality more complicated.

The results of experiments to determine the effect of substrate upon the reactivation show that substrate interferes with the action of the oxime on the inhibited enzyme. The effect is more marked the lower the concentration of oxime. The substrate does not, however, prevent the reactivation from occurring but it proceeds at a reduced rate, and in the case of the equilibrium type of reactivation the same position is eventually attained as that resulting from the addition of oxime prior to the substrate.

The studies on the rate of formation of a form of the inhibited enzyme incapable of being reactivated were made at only one pH value. At pH 7.4 and 25° C the rate of transformation was comparatively slow for both enzymes with the inhibitors used in this work. It appears, however, to be fastest with sarin-inhibited eel cholinesterase.

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EFFECTS OF GROWTH HORMONE ON METABOLIC AND ENDOCRINE FACTORS IN HYPOPHYSECTOMIZED DOGS[1]

JAMES CAMPBELL, LEO CHAIKOF, GERALD A. WRENSHALL, AND REUBEN ZEMEL

Abstract

Hypophysectomy in dogs caused reductions in: the level of sugar in the blood; the volume % of packed red blood corpuscles; the rate of clearance of bromsulphthalein; the weight of the pancreas; the amount of insulin extractable from the pancreas; and the weights of the kidneys, heart, and adrenal glands. The glucose tolerance of hypophysectomized dogs was significantly less than that of normal dogs.

The administration of growth hormone during 5 days to hypophysectomized dogs caused: increase of blood sugar but not to diabetic levels; elevation and prolongation of the glucose tolerance curve; increased erythrocyte sedimentation rate; reduced volume % of red blood corpuscles; restoration of bromsulphthalein clearance to normal; reduction of the insulin extractable from the pancreas to 60% of the pretreatment level; increases in the weight, total lipid, dry fat-free residue, and water of the liver; and restoration of the kidney weight and lipid content to normal. Little or no glycosuria or ketonuria and no anorexia occurred under these conditions.

The same treatment of normal dogs with growth hormone caused: increase of blood sugar to diabetic levels; elevation and prolongation of the glucose tolerance curve; increased erythrocyte sedimentation rate; decreased volume % of packed red blood corpuscles, glycosuria, ketonuria, albuminuria, and anorexia; great decrease in the amount of insulin extractable from the pancreas to 6% normal; increase in the total lipid, dry fat-free residue, and water of the liver, increase in the weight and lipid of the kidney; and increase in the weight of the ventricles of the heart. The bromsulphthalein clearance was not altered. The physiological significance of these findings is discussed, particularly the production by growth hormone of more pronounced diabetes and greater reduction of insulin extractable from the pancreas in normal than in hypophysectomized dogs.

Introduction

The metabolic alterations that follow hypophysectomy in dogs include decreased metabolic rate, tendency to hypoglycaemic crises when fasting, and increased sensitivity to insulin (1). Hypophysectomized dogs have a somewhat reduced tolerance to glucose, which is further lowered by the administration of growth hormone (2). They are also, according to deBodo and his colleagues (3), sensitive to the "toxic" effects of growth hormone.

In intact dogs, growth hormone can produce intense diabetes with great reduction of the insulin of the pancreas. Fattiness of the liver and kidneys accompany this diabetic state, and frequently signs of "toxicity" occur (4). We have therefore compared hypophysectomized and normal dogs, and their responses to growth hormone, under the same experimental conditions, measuring changes in the compositions of blood, urine, and tissues, and also testing liver function. Some of these findings have been described briefly (5).

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Methods and Materials

The blood sugar (6), erythrocyte sedimentation rate (ESR), volume % of red blood corpuscles (7), and urine sugar (8) were determined by the methods indicated. Rothera's test for ketone bodies was used. Albumin in urine was measured by the turbidity produced by sulphosalicylic acid in the final concentration of 2.5%, compared with that of a standard solution of bovine plasma albumin. Glucose tolerance was tested about 18 hours postprandially by injecting intravenously, within one minute, the equivalent of 1 g of glucose per kg of body weight. The solution contained 40% of glucose in saline and was injected at 37° C. Bromsulphthalein clearance was also tested 18 hours after a meal by the intravenous injection of solution equivalent to 10 mg of the dye per kg of body weight, and the determination of the dye remaining in the plasma after 15 and 30 minutes (9).

At sacrifice the dogs were anaesthetized by the intravenous injection of nembutal, were bled from a carotid cannula for several minutes, and samples of the tissues were quickly excised. The glycogen of the liver was determined (10) and the total lipid, the water, and the dry, fat-free residue of the liver, kidney, and ventricular muscle were determined by exhaustive extraction of 5-g samples with acetone, hot ethanol, and ether, followed by rectification of the crude lipids with petroleum ether, as described by Best, Lucas, Patterson, and Ridout (11). Small samples of the head, body, and tail of the pancreas were removed for histological examination (12, 13). The remainder of the organ, about 85%, was extracted as described by Labuschagne, Haessig, and Wrenshall (14) and the extract was assayed for insulin by the mouse-convulsion method.

Each dog was fed 500 g of horse meat daily, given as two meals at 10 a.m. and 4 p.m. The lipids were extracted from 11 samples of the horse meat by the method noted above (11) and the total nitrogen of the dry, fat-free residue was determined by Kjeldahl analysis. The mean values thus obtained were: total protein ($N \times 6.25$) = 17.8 g and total lipid 14.8 g per 100 g of fresh minced horse meat. While the hypophysectomized dogs ate rather slowly, they usually finished the meals within half an hour. The bovine growth hormone, Connaught Medical Research Laboratories, lot 200, had full growth-promoting activity; and contamination by other known pituitary hormones was so slight as to be negligible. It was injected subcutaneously twice daily, with the meals.

Experimental Procedures and Results

Adult dogs were hypophysectomized by the transbuccal method described by Markowitz and Archibald (15). During healing the food intake was low and the body weight decreased but recovery, with regain of weight, appeared to be complete within two weeks. The metabolism of seven hypophysectomized dogs was studied during periods ranging from 45 to 105 days postoperatively, while the same measurements and tests were made concurrently on normal dogs. Following this pretreatment period, five of the hypophysectomized dogs and four intact dogs were each given 1 mg of growth hormone per day for

5 consecutive days, then sacrificed 18 hours after the last injection. The controls for this treatment were two hypophysectomized and four normal dogs similarly injected with saline.

At the end of these experiments serial sections of the tissues about the site of operation were examined and the volume of the pituitary tissue was estimated by means of a calibrated scale. Very small fragments of *pars tuberalis* tissue remained adherent to the hypothalamus of eaeh of the operated dogs, and in one dog (No. 139) some *pars intermedia* also, but in no case was any *pars anterior* tissue found. The significance of differences tested according to Fisher (16), and standard errors (S.E.) of means are shown in the tables.

Sugar of Blood and Urine

As was expected from previous investigations (1), the mean blood sugar level of the hypophysectomized dogs was lower than that of the normals by 10 mg% during the pretreatment period of observation (Tables I and II).

TABLE I

Comparison of normal and hypophysectomized dogs

Venous blood samples were taken from normal (N) and hypophysectomized (H) dogs about 18 hours after feeding. The standard errors of the means, t and p are given (16). During the treatment period of 5 days, saline or growth hormone (GH), 1 mg per kg of body weight per day, was injected

		Pretreatment period			Treatment period		
State	Dogs Nos.	Body wt., kg	Mean blood sugar, mg%	Mean red blood corpuscle vol., %	Injection	Body wt. gain, %	Final red blood corpuscle vol., %
H	139	15.9	63	54	Saline	1.3	51
	165	9.0	42	46	Saline	5.3	42
	162	11.6	37	45	GH	5.2	38
	151	10.8	46	45	GH	6.5	36
	143	14.7	29	47	GH	5.4	39
	133	14.5	54	49	GH	6.2	44
	Means		45 ± 4.9	48 ± 1.4		5.8	39 ± 1.7
N	166	8.7	53	47	Saline	2.3	49
	177	14.1	55	53	Saline	0.8	52
	1	15.7	56	52	GH	5.1	52
	2	8.9	62	50	GH	7.9	42
	6	15.4	56	47	GH	4.6	45
	194	9.2	56	50	GH	8.5	43
	Means		56 ± 1.2	50 ± 1.0		6.5	45 ± 2.2
Tests of significance							
H vs. N	t		2.2	1.2			
	p		0.05	0.2			
H + GH vs. N + GH	t					2.3	
	p					0.05	

In the hypophysectomized animals, the range was greater and very low values were found, although hypoglycaemic reactions were not observed. In these dogs the urines were free of both sugar and ketone bodies, and the volumes were comparable, except that the urine volume of the hypophysectomized dog 162 was high throughout the experiment.

In the intact animals given growth hormone, the fasting blood sugar rose to diabetic levels after 3 days and remained high (Fig. 1). The average urine

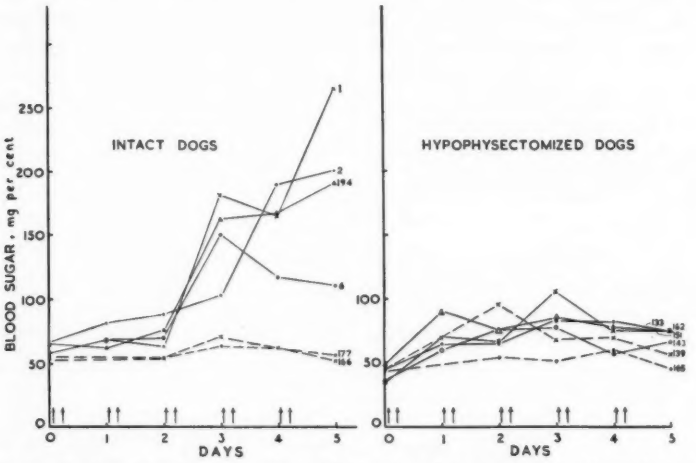

FIG. 1. Fasting blood sugar values of intact and hypophysectomized dogs given growth hormone, 1 mg per kg of body weight per day, solid lines; or saline, broken lines, at the injection times shown by the arrows.

volume increased and sugar was excreted by each dog from the third day on, range 3 to 60 g daily (Fig. 2). Ketonuria occurred in three of these dogs, i.e. in No. 1 from the third day on and in Nos. 2 and 194 on the last day. The growth hormone induced albuminuria in all the dogs. Before injection the daily excretion of protein was 6 to 16 mg per dog, and on the fourth day of injection was 17 to 137 mg, mean 105 mg. During the last 2 days of injection anorexia and lethargy were noted and vomiting occurred. These effects have been observed previously but with larger doses of growth hormone (**4**).

The blood sugar of the hypophysectomized dogs increased during the injections of growth hormone by amounts ranging from 38 to 51 mg%. The highest level reached, however, was only 106 mg% (Fig. 1). The urine volumes of three of these dogs did not change appreciably (Fig. 2); while in the dog with chronic polyuria, No. 162, the volume prior to injection was 1100 ml and on successive days during injection was 930, 1500, 1430, 1750, and 2000 ml per day. During the fourth day of injection, three of the hypophysectomized dogs excreted 2 to 3 g of sugar (Fig. 2). However, the two control hypophysectomized dogs, given injections of saline, also excreted this amount of sugar at the same time. A glucose tolerance test had been performed on these test and control hypophysectomized dogs early during the fourth day, so that their sugar

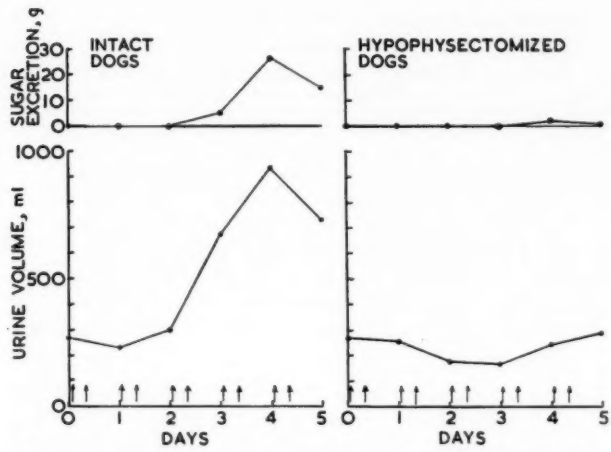

FIG. 2. The mean sugar excretions and urine volumes of intact and hypophysectomized dogs given growth hormone (1 mg per kg of body weight per day) at the times indicated by the arrows. The urine volumes of the hypophysectomized dog No. 162, with polyuria, have not been included.

excretion was probably due to the extra sugar load. The result indicates that in hypophysectomized dogs growth hormone had a small effect on glycosuria as compared to the effect in intact dogs, and also that the kidney threshold for glucose may be reduced after hypophysectomy. During the last day only one hypophysectomized dog excreted 2 g of sugar. Ketonuria of mild degree occurred in only one of these hypophysectomized dogs (No. 133) given growth hormone. No upset, such as anorexia, vomiting, or lethargy, was noticed in them.

It is seen that the brief treatment with growth hormone elicited diabetes in intact dogs, with hyperglycaemia, glycosuria, ketonuria, and albuminuria but in hypophysectomized dogs so treated these occurred in only minor degree.

Glucose Tolerance

The glucose tolerances of normal and hypophysectomized dogs both in the untreated state and after injections of growth hormone for 3 days are shown in Table II. The net disappearance of sugar from the blood during the initial phase was roughly exponential and the net rate constant (K) was obtained for each animal by equation 1:

$$K = 2.30 \frac{(\log_{10} G_1 - \log_{10} G_2)}{t_2 - t_1}$$

where G_1 and G_2 are the sugar concentrations at times t_1 and t_2 respectively.

It has been reported previously by Biasotti (17), Foglia and Potick (18), and deBodo and colleagues (19) that hypophysectomy decreased tolerance to glucose. The present experiments confirm this observation. In the hypophysectomized dogs the blood sugar concentrations were significantly above normal after giving glucose, although the initial level was below normal by 10 mg%.

TABLE II

Glucose tolerance tests

The glucose tolerance of the intact (N) and hypophysectomized (P) dogs was tested by the intravenous administration of 1 g of glucose in saline per kg of body weight during the treatment period, and in the same dogs after 3 days of injection of growth hormone (GH), 1 mg per kg of body weight per day

Dogs		Mean blood sugar after glucose, mg % ± S.E.						
State	No.	0 min	15 min	30 min	60 min	120 min	180 min	240 min
N	7	61± 3.08	230±10.5	125±20.2	69± 4.85	72± 2.42	70± 2.98	71± 2.79
H	7	51± 4.04	259±17.2	213± 9.31	149±10.5	70±12.6	56± 4.91	50± 3.52
N + GH	4	151±17.6	323±15.7	283±13.9	216± 7.04	158± 7.2	136± 5.79	100±10.7
H + GH	4	88± 6.2	294± 9.1	258± 6.5	210±13.1	151±15.3	117±14.7	95± 6.77
		Tests of significance						
N vs. H	t	2.0	1.7	4.0	7.0	0.15	2.4	4.7
	p	0.05	0.1	0.005	0.001	N.S.*	0.04	0.001
N vs. N + GH	t	6.7	5.1	5.4	17.6	13.9	11.4	3.34
	p	0.001	0.001	0.001	0.001	0.001	0.001	0.001
H vs. H + GH	t	5.2	1.4	3.4	3.6	4.0	4.8	6.6
	p	0.001	0.2	0.01	0.01	0.005	0.001	0.001
N + GH vs. H + GH	t	3.4	1.6	1.6	0.4	0.4	1.2	0.4
	p	0.02	N.S.*	N.S.	N.S.	N.S.	N.S.	N.S.

*Not significant.

The value of K was significantly reduced to one-third of normal and the return of the blood sugar to the initial level was delayed (Table III).

TABLE III

Rate constants for net disappearance of glucose

The initial rate constant (K) for the net disappearance of glucose from the blood of each dog given glucose intravenously was calculated by equation 1 and averaged by groups. The group symbols are: normal (N), hypophysectomized (H), growth hormone injected (GH)

Dog, state	No. of dogs	Interval from the time of injection of glucose, min	K ± S.E.	Approx. time of return to initial blood sugar concn., min
N*	7	15–30	0.0434±0.00596	60
H	7	15–60	0.0133±0.00145	180
N + GH	4	15–60	0.00864±0.000714	180
H + GH	4	15–60	0.00751±0.00143	240
		Tests of significance		
N vs. H		t	2.75	
		p	0.01–0.025	
N vs. N + GH		t	3.29	
		p	0.01	
H vs. H + GH		t	6.06	
		p	0.001	
N ± GH vs. H + GH		t	0.48	
		p	N.S.	

*The N group value of K for the 15- to 60- minute interval was 0.0263±0.00208, but since the rate slowed during 30–60 min, the rate during the 15- to 30- minute interval was used.

Treatment of these hypophysectomized dogs with growth hormone caused further great reduction in glucose tolerance. The initial blood sugar level was raised by 37 mg%, K was reduced to one-sixth of normal, and the return of the blood sugar to the initial concentration was prolonged (Table III).

The growth hormone injections also greatly reduced the glucose tolerance of the normal dogs; the blood sugar was initially at diabetic level, K was reduced to one-fifth of that in the same animals when untreated, and the return of the curve to the initial value was delayed (Table III). In these intact dogs given growth hormone the glucose tolerance curve continued to fall, after 2 hours, significantly below the initial level (Table II). The growth hormone reduced the glucose tolerance of the normal and the hypophysectomized dogs to about the same extent.

The highest elevation of the glucose tolerance curve (blood sugar values after the injection of glucose less the initial value) occurred in the hypophysectomized dogs given growth hormone. It was lower, in decreasing order, in the untreated hypophysectomized dogs, in the normals given growth hormone, and in the untreated normals.

Bromsulphthalein Clearance

The clearance of bromsulphthalein from the blood plasma was delayed in the hypophysectomized dogs. The mean concentrations of the dye in the plasma at 15 and 30 minutes after intravenous injection significantly exceeded the normal values by 100 and 85% respectively (Table IV). The administration of growth hormone for 4 days restored the bromsulphthalein clearance of these hypophysectomized dogs to that of normal dogs (Table IV). This effect occurred in each operated animal and the differences between means before and after the treatment were significant. In normal dogs the bromsulphthalein clearance rate was not significantly altered by the administration of growth hormone.

Erythrocyte Sedimentation Rates, etc.

In both hypophysectomized and normal dogs the ESR increased when growth hormone was administered, the change being perceptible within a day of the first injection and definite within two days (Fig. 3). This is one of the earliest and most sensitive of the responses of fed dogs to growth hormone, and was found to be associated with rise in plasma fibrinogen (4). The preinjection sedimentation rates of two of the hypophysectomized dogs were high. This may be related to their lesser volume % of erythrocytes (Table I), noted previously by Houssay, Royer, and Orias (20). The treatment with growth hormone reduced the hematocrit readings of the hypophysectomized and the normal dogs (Table I). This effect was found to be due to increase in the volume of the plasma (4). Body weight increased during the injections of growth hormone (Table I).

Pancreas

The amounts of insulin extractable from the pancreas (AIEP) are shown in Table V. The average AIEP of the hypophysectomized dogs was less than that of the normal dogs by 11% when calculated as units per gram of pancreas, or by 38% when calculated per kilogram of body weight. This difference, though not significant statistically, is in agreement with the results of Taylor, Essex, and Wrenshall (21). After the hypophysectomized dogs had received growth hormone for 5 days, the AIEP was significantly decreased to 60% of

TABLE IV

Bromsulphthalein clearances

The bromsulphthalein clearances of normal (N) and of hypophysectomized (H) dogs were tested by the intravenous injection of 10 mg of the dye per kg of body weight. The tests were performed before and in the same dogs after the administration of growth hormone for 4 days (1 mg per kg of body weight per day) or of equivalent volumes of saline

State of dogs	Dogs Nos.	Pretreatment period, % retention of dye		Treatment period, % retention of dye	
		15 min	30 min	15 min	30 min
		Growth hormone			
N	1	11.4	6.0	13.3	8.7
	2	4.0	2.8	5.9	4.0
	6	11.1	6.2	10.5	5.7
	194	5.1	2.5	3.7	0
Means				8.4	4.6
S.E.				±2.37	±1.82
		Saline			
N	177	7.8	2.3	11.0	4.2
	166	7.6	2.0	5.9	3.5
	4	4.0	2.0		
Means		7.3	3.4	8.5	3.9
S.E.		±1.17	±0.71	±2.5	±0.35
		Growth hormone			
H	133	21.8	7.8	8.9	5.3
	143	18.0	7.0	4.9	2.7
	151	9.8	4.2	4.9	2.2
	162	16.0	10.0	10.9	4.6
Means				7.4	3.7
S.E.				±1.5	±0.74
		Saline			
H	139	10.9	4.7	24.3	9.1
	165	9.1	3.6	9.5	4.2
	178	15.5	6.5		
Means		14.4	6.3	16.9	6.7
S.E.		±1.78	±0.85	±7.4	±2.4
		Tests of significance			
N vs. H	t	3.38	2.42		
	p	0.005	0.02–0.05		
H vs. H + GH	t	2.64	2.02		
	p	0.02–0.05	0.05		
N vs. N + GH	p	N.S.	N.S.		

that of the hypophysectomized controls. In contrast, the growth hormone caused a very great decrease in the AIEP of the intact dogs, to about 6% of that of their controls in the same time. This difference was highly significant. This agrees with the results of previous studies in which, however, larger doses of growth hormone were given to intact dogs (4). After the treatment with growth hormone the average AIEP of the intact dogs was only 12 to 15% of that of the hypophysectomized dogs so treated (Table V). This difference was also highly significant. Thus growth hormone in a few days reduced the insulin obtained from the pancreas, but the decrease was of much greater magnitude in the intact than in the hypophysectomized dogs.

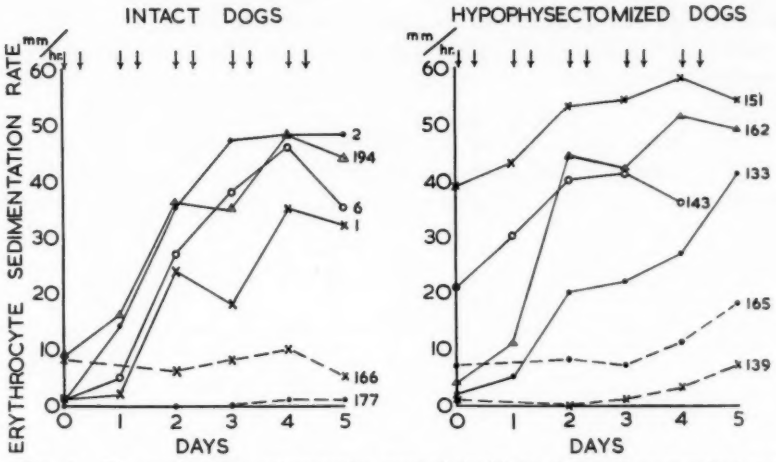

FIG. 3. The erythrocyte sedimentation rates of the blood of hypophysectomized and normal dogs (numbered) given injections of growth hormone (solid lines) or of saline (broken lines), as indicated by the arrows.

TABLE V

Amount of insulin extractable from the pancreas

The test normal (N) and hypophysectomized (H) dogs were given 1 mg of growth hormone (GH) per kg of body weight per day for 5 days, while the controls were similarly injected with saline. The pancreases were taken 18 hours after the last injection. The standard errors of the means and values of t and p (16) are given

State of dogs	Injections	No. of dogs	Pancreas: Weight, fresh g per kg of body weight	Pancreas: Insulin, units per g pancreas	Pancreas: Insulin, units per kg of body weight
N	Saline	4	1.90±0.17	3.85±0.35	7.35±0.92
N	GH	4	2.01±0.31	0.26±0.050	0.47±0.025
H	Saline	3	1.16±0.089	3.44±0.276	5.28±0.25
H	GH	4	1.44±0.12	2.14±0.16	3.09±0.36
			Tests of significance		
N vs. H	t		3.46	0.86	1.87
	p		0.02	N.S.*	0.2
N vs. N + GH	t		0.31	10.2	7.4
	p		N.S.	0.001	0.001
H vs. H + GH	t		1.78	4.06	4.58
	p		0.2	0.01	0.01
N + GH vs. H + GH	t		1.70	11.0	7.2
	p		0.2	0.001	0.001

*Not significant.

The beta cells of the pancreatic islets of the normal dogs stained by Wilson's method (13) had the usual density of the characteristic cytoplasmic granules, while in the hypophysectomized dogs the granulation was estimated to be 25% less. In the dogs given growth hormone loss of granulation occurred, in degree roughly parallel to the extractable insulin values, i.e. especially in the

intact (75% loss), but also in the hypophysectomized dogs (37% loss). These losses were due to disappearance of granules from cells, not to reduction in the number of beta cells.

Adrenals

The adrenal glands of the hypophysectomized dogs were about one-half the normal weight. The cortex was much thinned and the medulla was relatively prominent (Table VI). After injections of growth hormone for 5 days the

TABLE VI

Adrenal and thyroid weights

The conditions of the experiments are given in Table V

State of dogs	Injections, daily for 5 days	No. of dogs	Adrenal glands, mean wet wt.	Thyroid glands, mean wet wt.
			g per kg of body weight	
N	Saline	4	0.104 ±0.0075	0.083 ±0.0073
N	GH	4	0.140 ±0.009	0.075 ±0.014
H	Saline	3	0.057 ±0.0087	0.060 ±0.0040
H	GH	4	0.060 ±0.019	0.070 ±0.0026
		Tests of significance		
N vs. H		t	3.82	1.9
		p	0.01–0.02	N.S.*
N vs. N + GH		t	3.02	0.4
		p	0.02–0.05	N.S.
N + GH vs. H + GH		t	3.82	
		p	0.01	N.S.
H vs. H + GH		t	1.54	
		p	N.S.	N.S.
N vs. H + GH		t	2.18	2.4
		p	0.05	0.05

*Not significant.

adrenal gland weights were higher than in the controls, this difference being significant at the 2 to 5% level in the normal, but not in the hypophysectomized dogs. The growth hormone caused marked changes in the *zona glomerulosa*, including cellular hypertrophy and reduction of the sudanophilic staining reaction. These changes are suggestive of increased activity. The weights of the thyroid glands, however, did not differ markedly in these animals (Table VI).

Liver

The liver, kidney, and heart weights and compositions are given in Table VII. The control hypophysectomized and normal dogs did not differ in liver weight and composition. The injections of growth hormone for 5 days caused enlargement of the livers of both the normal and the hypophysectomized dogs, with massive accumulation of fat which was strikingly apparent on surface inspection by the yellow color. Increased friability was also evident. This effect was greatest in the intact dogs whose livers were almost twice the weight

TABLE VII

Weights and total lipids of organs

Intact (N) and hypophysectomized (H) dogs were given growth hormone (GH), 1 mg per kg of body weight daily for 5 days, and control dogs were similarly injected with saline (S). The values, with the standard errors, are for the mean weights of the organs and for the total lipid (TL), for dry, fat-free solids (DFFS), and for water (W) of the organs, and are all given on the basis of g per kg of final body weight. From these data the values may also be calculated per 100 g of fresh tissue or per 100 g of dry, fat-free solids of the organ

Dogs			Liver				Kidney				Heart, ventricles			
State	Injection	No.	Wt., fresh	TL	DFFS	W	Wt., fresh	TL	DFFS	W	Wt., fresh	TL	DFFS	W
N	S	4	23.7 ± 1.17	1.35 ±0.11	5.52 ±0.28	16.9 ± 0.81	4.37 ±0.37	0.224 ±0.057	0.713 ±0.074	3.41 ±0.17	6.68 ±0.29	0.257 ±0.071	1.22 ±0.11	5.20 ±0.34
N	GH	4	40.7 ± 2.52	7.98 ±1.62	7.04 ±0.59	25.7 ±2.36	6.82 ±0.92	0.486 ±0.099	1.05 ±0.16	5.28 ±0.71	7.34 ±0.65	0.396 ±0.030	1.41 ±0.18	5.71 ±0.52
H	S	2	26.9	1.28	6.27	19.3	3.44	0.154	0.514	2.76	4.77	0.189	0.781	3.81
H	GH	4	39.2 ± 6.34	4.97 ±1.52	7.70 ±1.13	26.6 ±4.05	4.40 ±0.24	0.223 ±0.017	0.705 ±0.023	3.47 ±0.25	5.09 ±0.39	0.257 ±0.039	0.861 ±0.088	3.99 ±0.26

of those of the normal controls, and contained five to six times more total lipid, 27% more total protein (dry fat-free solids), and 50% more water per kg of body weight. These differences were all highly significant. The total lipid of the liver as per cent of fresh tissue was increased 3.5-fold by the injections of growth hormone; means 19.8 and 5.7 in the test and control dogs respectively. This increase in liver lipid as per cent of the fresh tissue was not as great as the increase per kilogram of body weight because the liver coincidently enlarged under the influence of growth hormone. The corresponding values of total lipid per gram of dry fat-free residue of the liver were 1.13 and 0.24 g respectively. This confirms previous findings that growth hormone causes remarkable enlargement of the liver in dogs, with increases in total lipid, protein, and water contents in a few days (4).

In hypophysectomized dogs given growth hormone the total lipid of the liver was increased to 390% of the control value, on the basis of body weight; while lesser increases occurred in the dry fat-free residue and the water of the organ (23 and 38% respectively). The total lipid of the liver in the test and control hypophysectomized dogs averaged 12.7 and 4.8% of the fresh tissue, respectively, or 0.65 and 0.20 g per g of dry fat-free residue.

The gain in water by the liver, per kilogram of body weight, could be expected from the gain in the dry, fat-free residue, which consisted chiefly of protein (about 83%). In the liver tissue of both the intact and hypophysectomized dogs given growth hormone, however, the water per gram of dry fat-free solids (3.65 and 3.45 g respectively) was higher than in their controls (3.06 and 3.07 g respectively). The difference (significant for the non-operated dogs) cannot be attributed to changes in glycogen, for the mean glycogen concentrations in the livers of the intact and hypophysectomized dogs given growth hormone and of those given saline were not obviously different, being 6.5, 5.9, 6.5, and 6.2% respectively. The increased accumulation of fat is not likely to be accompanied by appreciable amounts of water. The data indicate that either the protein laid down in the liver by this short-term action of growth hormone bound more water per gram than the total protein of the liver in the control animals or that another influence, possibly increased salt, caused retention of water.

Kidneys

In the hypophysectomized dogs the weights and the lipid, protein, and water contents of the kidney were below normal. These were restored to approximately normal values by the administration of growth hormone (Table VII). Growth hormone enlarged the kidneys of the normal dogs, due to increases in lipid (doubled), protein, and water per kilogram of body weight.

Heart

The weights, and the lipid and protein contents per kilogram of body weight of the ventricles of the hearts from the hypophysectomized dogs were below normal, and were increased, but not to normal, by the growth-hormone injections. In the intact dogs given growth hormone, the ventricle weight, lipid and protein content per kilogram of body weight, and lipid per 100 g of fresh tissue were above the normal control values.

Discussion

Growth hormone produced a definitely diabetic state, characterized by hyperglycaemia, polyuria, glycosuria, and ketonuria in normal dogs but in hypophysectomized dogs so-treated the corresponding changes were smaller or absent. The treatment also reduced the insulin extractable from the pancreas much more in the intact than in the hypophysectomized dogs. Obviously, the growth hormone produced disparity between the rate of formation of insulin versus the rate of removal of insulin from the pancreas, the disparity being of greater magnitude in the intact animals. In seeking an explanation of these findings it is necessary to consider the functional activities of the islets of Langerhans.

The investigations of Milman and Russell (22), Milman, DeMoor, and Lukens (23), Bennett (24), and Randle and Young (25) indicate that growth hormone may increase the rate of secretion of insulin from the pancreas during early phases of treatment. This is indicated also by contrasting the responses to growth hormone of normal dogs with those of depancreatized and metasomatotrophin-diabetic dogs while they are receiving their usual maintenance, or increased, doses of insulin. Relatively small doses of the growth hormone greatly increased the sugar excretion and raised the requirement for insulin of the diabetic dogs severalfold, whereas much larger doses and longer periods of injection were required to elicit diabetes in normal dogs (26, 27). This resistance of normal animals to the diabetogenic effects of growth hormone can be attributed to enhanced secretion of insulin, called forth in response to increased demands for insulin created by growth hormone. The early reduction of the insulin of the pancreas of dogs by growth hormone appears to be caused by a greatly increased rate of secretion of insulin rather than a marked decrease in the rate of formation of insulin. It should be emphasized, however, that it is not implied that the rate of formation of insulin may be diminished by growth hormone — the converse could well be expected at the start of growth hormone injections.

Hypophysectomy reduces general metabolism and diminishes the activities of the thyroid, adrenal, and sex glands (28). It could therefore be expected that the metabolic responsiveness of hypophysectomized dogs may be diminished, so that in them the growth hormone may increase the demands for insulin by the extrapancreatic tissues to a lesser extent than in intact animals. This appears to be supported by the finding that growth hormone increased the rates of transfer of glucose into and out of the plasma to a greater extent in normal than in hypophysectomized dogs (29). Consequently, in hypophysectomized animals given growth hormone, less extra secretion of insulin and smaller depletion of pancreatic insulin would be predicted than in similarly treated normal animals.

This problem is obviously related to that of the rate of secretion of insulin in the hypophysectomized animal. Houssay, Foglia, Smyth, Rietti, and Houssay (30) tested the liberation of insulin from excised pancreases by grafting them to the circulation of depancreatized dogs. The pancreases taken from hypo-

physectomized and normal dogs did not differ in their blood sugar lowering effect in the recipients. Other results indicate the possibility, however, that in hypophysectomized dogs the secretion of insulin may be less than normal, e.g. hypophysectomized dogs are sensitive to insulin (1), in them the amount of insulin extractable from the pancreas (21) and the insulin activity of the plasma are reduced (25), and the rate of transfer of glucose through the plasma is subnormal (31).

The low rate of clearance of bromsulphthalein in hypophysectomized dogs is an indication that their liver function is less than normal, although probably adequate for their reduced metabolic activity. The effect of growth hormone in restoring their clearance rates to normal indicates that the hormone stimulated the functional activity of the liver. This was accompanied by enlargement of the organ due to great increases in total lipid, total protein, and water. Growth hormone did not appreciably alter the bromsulphthalein clearance of intact dogs, but caused even greater enlargement of the liver and greater increases in its lipid and protein than in the operated dogs. Thus it appears probable that the effect of the hormone on the liver may be greater in the normal. These relations suggest that although the hormone may produce a greater change in an index of metabolism in hypophysectomized than in normal animals, the possibility remains that the *total effect* may be greater in the former. Certain compensatory processes, for example, may become more active in normal than in hypophysectomized animals and so reduce the magnitude of the changes.

The reduced weights of the kidneys and the ventricles of the heart in hypophysectomized dogs are probably related to lesser metabolic demands in this state. Renal function of hypophysectomized dogs is subnormal, and is somewhat raised by treatment with growth hormone (32, 33). The restoration of kidney weight to normal level with increase in lipid content, and the partial restoration of the weight of the ventricles, by growth hormone may likewise be related to increased metabolic demands. In intact dogs the growth hormone caused enlargement and increased accumulation of lipid in the kidney and heart.

The indications that growth hormone increased cellular activity in the *zona glomerulosa* of the adrenal cortex will be studied further. The growth hormone preparation used had negligible adrenocorticotrophic activity when tested by its effect on the adrenal ascorbic acid of hypophysectomized rats.

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THE ISOLATION, IDENTIFICATION, AND PROPERTIES OF DINUCLEOTIDES FROM ALKALI HYDROLYZATES OF RIBONUCLEIC ACIDS[1]

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Abstract

The isolation, identification, and properties of dinucleotides from alkali hydrolyzates of RNA are described. Preparative methods for obtaining the alkali-labile dinucleotides which were found in our 18-hour hydrolyzates of RNA in 1 *M* potassium hydroxide are described, and it is noted that all of them (ApAp, ApGp, ApUp, GpAp, and GpCp) are not susceptible to pancreatic ribonuclease at the concentrations of the enzyme which are customarily employed for hydrolytic degradations. It has been found, however, that two of these dinucleotides (ApAp and ApUp) are susceptible to pancreatic ribonuclease at concentrations of the enzyme which are about five thousand times greater than those customarily employed. The rate constants of first order hydrolysis in alkali of ApAp, ApGp, ApUp, GpAp, and GpCp are found to be correspondingly lower than the rate constants for two dinucleotides which were *not* found in our 18-hour hydrolyzates, and which *are* susceptible to pancreatic ribonuclease. Furthermore, it is reported that the eight possible diribonucleotides which are not susceptible to pancreatic ribonuclease (ApAp, ApGp, ApCp, ApUp, GpGp, GpAp, GpCp, and GpUp) are found in roughly equivalent amounts in a 2-hour alkali hydrolyzate of a preparation of yeast RNA, and account for 80–90% of the dinucleotides present.

In addition to the alkali-labile dinucleotides found in alkali hydrolyzates of RNA, the isolation and preliminary characterization of five alkali-stable compounds are reported.

Introduction

It has often been reported (1, 2, 3, 4, 5, 6, 7) that in alkali hydrolyzates of RNA* there are compounds which are not mononucleotides and which may be classified arbitrarily as either non-ultraviolet-absorbing or ultraviolet-absorbing compounds. Davidson and Smellie found (1) that paper ionophoresis of the RNA fraction obtained by Schmidt–Thannhauser fractionation of tissue (8) indicated the presence of six non-mononucleotide, phosphorus-containing spots, only one of which absorbed ultraviolet light. Subsequent studies have indicated

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*The following abbreviations are used: The symbols A, G, C, and U refer to adenosine, guanosine, cytidine, and uridine respectively. A phosphate group is denoted by "p", and when written to the right of the symbol for a nucleoside the phosphate is esterified at the 3′-position of ribose; when written to the left of the nucleoside symbol the phosphate is esterified at the 5′-position of ribose. This nomenclature was originally proposed by Markham and Smith (Biochem. J. **52**, 558 (1952)) and has been extended somewhat in this paper so that when the symbol "p" is written to the right of the symbol for a ribonucleoside, and represents a singly esterified phosphate group, it is intended to represent a mixture of 2′ and 3′ isomeric forms. Where a distinction is to be made between the 2′- and 3′-isomers, the numeral is inserted immediately preceding the symbol "p". The insertion of the letter "c" immediately preceding the symbol "p" indicates a cyclic 2′,3′-phosphate group. Thus Acp represents adenosine 2′,3′-phosphate and ApA2p represents adenylyl adenylic acid with a singly esterified phosphate group on position 2′ of ribose.

The terms S-terminal and P-terminal are used to designate the end groups of oligonucleotides. The S-terminal group is the nucleotide residue which does not bear a singly esterified phosphate group. The P-terminal group is the nucleotide residue which bears a singly esterified phosphate group.

The symbols RNA and RNase refer to ribonucleate and pancreatic ribonuclease, respectively.

The abbreviation DEAE-acetate is used as a contraction for DEAE-cellulose column in the acetate form.

that three of the compounds were phospho-serine, phospho-inositol, and phospho-glycerol (9). Logan *et al.* found (2) that about half of the phosphorus of the RNA fraction obtained by Schmidt–Thannhauser fractionation of brain tissue was diphosphoinositide. When the RNA fraction obtained by Schmidt–Thannhauser fractionation of chick embryo muscle was subjected to anion exchange chromatography on Dowex-1-acetate, Cohen found (5) that, after elution of the mononucleotides, a fraction remained on the column which required a very strong eluting agent (sulphate was used) for its removal. This fraction contained only 5% of the ultraviolet absorption (at 260 mμ) of the original hydrolyzate but accounted for 25% of the phosphorus. It was thought that the fraction might be a mixture of unhydrolyzed oligonucleotides (maximum for ultraviolet absorption was 260 mμ), and some of the compounds originally reported by Davidson and Smellie which were bound to the protein component of the acid-insoluble residue and liberated during the alkali hydrolysis of the RNA.

Allen *et al.* have reported a number of compounds in alkali hydrolyzates of RNA which absorb ultraviolet light at 260 mμ and are detectable by paper chromatography of the neutralized hydrolyzates (3, 4). Potter and Dounce reported that alkali hydrolyzates of RNA isolated from various sources, when subjected to anion exchange chromatography, yielded a postmononucleotide fraction (eluted from anion exchange resins after the mononucleotides) which accounted for from 5% (yeast) to 38% (pancreas) of the ultraviolet absorption of the original hydrolyzates (6). We attempted to repeat this work but were unable to verify the quantitative figures. In our hands, the postmononucleotide fraction rarely exceeded 5% of the ultraviolet absorption of 24-hour hydrolyzates, regardless of the source of the RNA. The presence of amino acids could be verified although quantitatively they amounted to only trace quantities when compared with the amount of bases present. We did, however, verify that the ultraviolet absorption of the fraction was primarily due to the presence of the bases adenine and guanine and that the postmononucleotide fraction of the RNA fraction obtained by Schmidt–Thannhauser fractionation of pancreas and liver was also predominantly purine in its base composition.

The methods of Cohen for anion exchange fractionation of RNA hydrolyzates (5) proved to be suitable for preparing the purine-rich postmononucleotide fraction. The use of Dowex-1-acetate and acetate buffers as employed by Cohen for anion exchange fractionation permitted the elution of the mononucleotides at a higher pH and under more controlled conditions than was possible with Dowex-1-chloride and eluents containing hydrochloric acid. Furthermore, the use of acetic acid – sodium sulphate solutions for elution of the postmononucleotide fraction permitted it to be eluted at relatively high pH's.

Preparations of RNA from yeast (10, 11) and liver (12, 13), following hydrolysis in 1 *M* potassium hydroxide for 24 hours and subsequent anion exchange fractionation, yielded a postmononucleotide fraction (fraction X) which was predominantly purine in its base composition. Chromatography on paper indicated that fraction X from different sources contained essentially

the same compounds with only quantitative variations. The RNA fraction from yeast, which Davis and Allen reported to be rich in their "fifth mononucleotide" (14), when hydrolyzed in 1 *M* potassium hydroxide for 24 hours gave the largest yield of postmononucleotide fraction, amounting to 9% of the original ultraviolet absorption of the hydrolyzate; the same preparation was completely hydrolyzed to the 5′-mononucleotides and very small amounts of the nucleoside diphosphates by purified snake venom phosphodiesterase. This observation in conjunction with de Lamirande's report (15) that extended digestion of RNA in alkali gave 98% hydrolysis suggested that some of the ultraviolet-absorbing components of the postmononucleotide fraction might be unhydrolyzed oligonucleotides. This view was substantiated when it was found that many of the ultraviolet-absorbing compounds detected by paper chromatography could be converted to mononucleotides upon reincubation for another 24 hours in 1 *M* potassium hydroxide at 26° C.

In order to identify the components of fraction X it was necessary to have a fairly rapid and large-scale procedure for preparing RNA. The method of Pain and Butler (13) proved to be the best available, and it was used in the initial experiments on the identification of the components of fraction X from calf liver RNA. The results have been verified in a parallel series of experiments using commercial preparations of RNA. The experiments were carried out originally on 24-hour hydrolyzates of RNA in 1 *M* potassium hydroxide, but the qualitative results were the same as those obtained for 18-hour hydrolyzates of the same samples. Since preparative methods for dinucleotides which are described in this paper are based on either 4-hour or 18-hour alkali digests of RNA, we shall report the details and results of 18-hour digests with the understanding that these results are true of 24-hour digests with only quantitative differences.

Although the work reported here is concerned with the identification and purification of dinucleotides in fraction X (which have phosphorus (P)/base ratios and P/ribose ratios of 1.0), the high P/ribose ratios of fraction X which vary from 1.1 to 1.9 suggest that there are phosphorus-containing compounds in the fraction which are separated from the alkali-labile dinucleotides during their purification. The pyrimidine nucleotides are not present in sufficient amounts to account fully for the high P/ribose ratios obtained in some cases although the fact that the pyrimidine nucleotides are not accounted for in ribose analyses does contribute somewhat to the high P/ribose ratios obtained. In this regard it should be pointed out that the sugar portion of the S-terminal group of the alkali-stable dinucleotides reported in this work may not react in the orcinol test.

A preliminary report of this work (7) suggested that there might be a correlation between RNase specificity and the inherent resistance of internucleotide linkages to OH^--catalyzed hydrolysis. This proposal was based primarily on the fact that the dinucleotides which had been identified in our alkali hydrolyzates (ApAp, ApGp, ApUp, GpAp, and GpCp) were presumably resistant to RNase. It was also found that a 2-hour alkali hydrolyzate of the RNA isolated from yeast (11) contained between 3 and 5% of each of the eight

RNase-resistant diribonucleotides whereas none of the eight diribonucleotides which are susceptible to RNase could be found in comparable quantity. A more intensive study of this possibility has led to the discovery that ApAp and ApUp* are susceptible to high concentrations of RNase but the failure to obtain significant hydrolysis of ApGp and GpAp under the same conditions indicates that generalizations are not yet possible.

Diuridylic and dicytidylic acids were prepared from polyuridylate and polycytidylate, respectively, in order to provide material for studying the rates of alkali hydrolysis of dinucleotides which were susceptible to RNase and which did not appear in significant amounts in the alkali hydrolyzates of RNA. It was found that, in accordance with their susceptibility to RNase, UpUp and CpCp were hydrolyzed by alkali much more rapidly than ApAp, ApGp, ApUp, GpAp, and GpCp.

Regardless of whether or not the inherent resistance of internucleotide linkages to OH^--catalyzed hydrolysis is important in the mechanism of RNase action, it would seem that the relatively low ratio of pyrimidine to purine bases in fraction X can be in large part attributed to the fact that the rate of hydrolysis of internucleotide linkages between pyrimidine nucleotides is greater than the rate of hydrolysis of internucleotide linkages between purine nucleotides. Diadenylic acid represented 25–30% of the total postmononucleotide fraction of our 18-hour hydrolyzates, and it seems likely that the compound which Smith and Allen called spot 5 in their paper (3) was also diadenylic acid.

In addition to the alkali-labile dinucleotides that have been found in the hydrolyzates, we have found five additional compounds, one of which appears to be guanosine 2′,5′- and/or 3′,5′-diphosphate, that are completely stable in 1 *M* potassium hydroxide. Four of the compounds (D_1, D_2, D_3, D_4) have not been identified but they may be related to those recently reported by Smith and Dunn (16) since they are completely stable in alkali and yield compounds with the properties expected of dinucleoside monophosphates when they are treated with prostatic phosphomonoesterase. One of the compounds, D_1, contains equimolar quantities of guanine and uracil, and another D_4, contains guanine as its only base. The mixture of D_2 and D_3 obtained from DEAE-cellulose columns contains 0.85 mole of adenine and 0.12 mole of cytosine per mole of guanine.

Experimental

Materials

RNA was prepared from yeast (10, 11) and from liver (12, 13). Commercial RNA was obtained from General Biochemicals and Schwarz laboratories.

Adenosine 2′,5′-diphosphate and adenosine 3′,5′-diphosphate were prepared from triphosphopyridine nucleotide and coenzyme A, respectively, by modifications of the procedure of Wang *et al.* (17).

Pancreatic ribonuclease (RNase) was obtained as a crystalline, protease-free preparation from Worthington Biochemicals.

*For purposes of brevity it will be assumed throughout this paper that ApAp and ApUp are not susceptible to pancreatic ribonuclease with the understanding that they can be degraded with excessively high concentrations of the enzyme.

Prostatic phosphomonoesterase was obtained from Professor G. Schmidt. The addition of 0.10 ml of the preparation to 2.5 μmoles of diadenylic acid dissolved in 4 ml of 0.2 *M* sodium acetate buffer, pH 5.4, caused complete hydrolysis of singly esterified phosphate in 4 hours at 37° C.

Polynucleotide phosphorylase was obtained from Professor S. Ochoa. The addition of 0.05 ml of the preparation to 50 mg of cytidine 5′-diphosphate dissolved in 2 ml of 0.15 *M* TRIS (Tris(hydroxymethyl)aminomethane) buffer at pH 8.1, containing 0.01 *M* magnesium chloride and 0.002 *M* EDTA (ethylenediamine tetraacetic acid), gave 25 to 30 mg of polycytidylate in 24 hours at 30° C.

Purified phosphodiesterase was prepared from Russell viper venom by the method of Hurst and Butler (18) and further purified by the method of Sinsheimer and Koerner (19). The addition of 0.50 ml of the preparation to 3 ml of 1% RNA solution buffered at pH 9.3 with 0.2 *M* DIOL (2-amino,2-methyl propanediol-1,3) buffer, caused 35% hydrolysis to mononucleotides in 18 hours with no detectable release of inorganic phosphate.

Rye-grass enzymes were prepared by the method* of Freeman and Butler (20) and nucleotidase activity was inhibited with oxalate unless otherwise indicated.

Dowex-1-X10, 200–400 mesh, was purchased from Dow Chemical Company of Canada.

DEAE-cellulose, type 20, reagent grade, was purchased from Brown Company (Berlin, New Hampshire, U. S. A.). Earlier preparations which gave very slow flow rates were mixed with equal volumes of Whatman reagent grade cellulose powder in order to achieve flow rates comparable with those obtained with the large particle size material now available.

Darco G-60 charcoal was washed by a procedure which consisted of heating a suspension of 400 g of the commercial charcoal in 1 liter of 2 *M* acetic acid for 2 hours at 100° C, filtering, and washing the charcoal at room temperature successively with 5 liters of water, 3 liters of ammoniacal ethanol, and 5 liters of water. The ammoniacal ethanol solution was made by mixing 500 ml of ethanol with 430 ml of water and 18 ml of concentrated ammonia.

Methods

Solvent Systems Used for Paper Chromatography

Solvent (*a*).—A solvent containing isopropanol, ethanol, and aqueous tartrate buffer has been used for chromatography of the compounds investigated in this work. This is used with paper pretreated with the buffer and with careful control of the atmosphere within the chromatographic unit. A detailed description of this and other new systems will be published shortly (Moscarello, M., Lane, B. G., and Hanes, C. S. In preparation.).

Solvent (*b*).—78 ml of saturated ammonium sulphate solution + 20 ml of 1 *M* sodium acetate solution + 2 ml of isopropanol. Equilibration was unnecessary and the solvent was run for 8 hours (21).

*The method is essentially the one originally reported by Shuster and Kaplan (J. Biol. Chem. **201**, 535 (1953)).

Solvent (*c*).—75 ml of ethanol + 30 ml of 1 *M* ammonium acetate, pH 7.5. The paper was equilibrated for 2 hours with the vapor of the solvent prior to a 20-hour run (22).

Solvent (*d*).—75 ml of ethanol + 30 ml of 1 *M* ammonium acetate saturated with boric acid and pH adjusted to 7.5 with ammonium hydroxide. The paper was equilibrated for 2 hours with the vapor of the solvent prior to a 20-hour run (20).

All paper chromatograms were developed using Whatman No. 3 paper which had been washed by the method of Connell *et al.* (23).

Column Chromatographic Techniques

Chromatography on Dowex-1-acetate was carried out by the method of Cohen (5). Modifications which have been introduced are given in detail under "Preparation of fraction X".

The description of methods used for chromatography on DEAE-cellulose (24) is given under "Preparation of fraction X".

Adsorption on Charcoal

The details for desalting by adsorption on and elution from charcoal have been published (25). The eluates were evaporated to dryness at 37° C in a "flash evaporator" and the residues were dissolved in water to give clear, colorless solutions. The aqueous solutions of the nucleotide materials had a pH of about 5.5. All fractions eluted from ion exchange columns were routinely desalted in this way. Recoveries from the charcoal adsorption procedure were usually 80–90% although trinucleotides and compounds containing the base guanine often required very large volumes for elution. It should be pointed out that the compounds investigated in this work have been found in the expected amounts in alkali hydrolyzates of RNA which have been neutralized and chromatographed on paper in solvent (*a*) without being placed in contact with either anion exchange resins or charcoal. This indicates that the compounds are present in alkali hydrolyzates and that their occurrence is independent of the preparative methods used. The small amounts of dinucleoside monophosphates which were sometimes found probably resulted from slight dephosphorylation caused by the preparative methods.

Analytical Methods

Purine bases and pyrimidine nucleotides were measured by the method of Markham and Smith (26), and ribose was estimated by the method of Albaum and Umbreit (27). Phosphorus was estimated by the method of King (28).

The following five methods of degradation were employed to identify the dinucleotides found in our alkali hydrolyzates of RNA.

Method (*a*).—A solution of the dinucleotide containing about 5 μg of phosphorus in 0.045 ml was mixed with 0.025 ml of 1.0 *M* sodium acetate buffer, pH 5.4, and 0.005 ml of prostatic phosphomonoesterase, and incubated at 37° C for 4 hours. After this time, 0.015 ml of 5 *M* potassium hydroxide was added and the digest was incubated for 24 hours at 26° C. The digests were neutralized with 3 *M* perchloric acid and applied to Whatman No. 3 paper for chromatographic identification of the products using solvents (*b*) and (*c*).

Method (*b*).—A solution of the dinucleotide containing about 5 μg of phosphorus in 0.045 ml was mixed with 0.045 ml of 1.0 *M* sodium acetate buffer, pH 5.4, and 0.045 ml of rye-grass nuclease, and incubated at 37° C for 2 hours. The rye-grass nuclease preparation had a concentration of 1.0 mg/ml on a dry weight basis, and much of the 3′-nucleotidase contamination had been removed by treatment with 0.06 *M* oxalate (20). The products of digestion were identified by paper chromatographic analysis using solvents (*b*) and (*d*).

Method (*c*).—A solution of the dinucleoside monophosphate containing about 5 μg of phosphorus in 0.20 ml was mixed with 0.20 ml of 0.2 *M* DIOL buffer, pH 9.3, and 0.20 ml of snake venom phosphodiesterase, and incubated at 37° C for 2 hours. The products of digestion were identified by paper chromatography using solvents (*b*) and (*d*).

Method (*d*).—The procedure was the same as method (*c*) except that the dinucleotide was used as substrate and the incubation was extended to 24 hours.

Method (*e*).—A solution of the dinucleotide containing about 5 μg of phosphorus in 0.20 ml was mixed with 0.20 ml of 0.1 *M* TRIS buffer, pH 7.5, and 0.20 ml of pancreatic RNase (1 mg/ml), incubated at 37° C for 24 hours and the products were identified by paper chromatography using solvents (*b*) and (*d*).

All chromatographic identifications were supplemented by chromatography of the digestion products on paper using solvent (*a*), which indicated the same products found when other solvents were used except that nucleosides and cyclic uridylic acid ran off the paper during the standard run.

Preparation and Fractionation of Fraction X

The following description is for a large-scale fractionation procedure which can be used to obtain milligram quantities of the dinucleotides found in fraction X. Since hydrolyzates of commercial samples of RNA contained all of the ultraviolet-absorbing compounds found in the hydrolyzates of calf liver RNA prepared by the methods of Kay and Dounce (12) and Pain and Butler (13), and all of the ultraviolet-absorbing compounds found in the hydrolyzates of yeast RNA prepared by the method of Crestfield *et al.* (11), many of the column chromatograms described will apply to a series of experiments carried out on commercial RNA (General Biochemicals and Schwarz preparations of RNA gave nearly identical results). Commercial RNA and yeast RNA yielded pGp in addition to the dinucleotides although pGp was never found in hydrolyzates of liver RNA.

(*i*) *Hydrolysis of RNA.*—RNA (10 g) was hydrolyzed in 500 ml of 1 *M* KOH for 4 or 18 hours at 26° C, cooled to 2° C and brought to pH 2 with 72% perchloric acid. The mixture was centrifuged for 2.5 minutes in a refrigerated centrifuge at 2000 r.p.m. to remove the potassium perchlorate and any acid-precipitable material. The supernatant solution was adjusted to pH 7 with 0.5 *M* potassium hydroxide.

(*ii*) *Fractionation of hydrolyzates on Dowex-1-acetate.*—A 25 cm × 3.3 cm column of commercial Dowex-1 resin was cycled three times with 2 liters of 2 *M* sodium hydroxide and 2 liters of 2 *M* acetic acid. The separation of frac-

tion X could be achieved by either of the following methods: (A) The neutralized hydrolyzate was charged on a Dowex-1-acetate column (which had been cycled and subsequently washed with carbon-dioxide-free water), the cytidylic and adenylic acids were chromatographically eluted with 1.3 *M* acetic acid – 0.01 *M* sodium acetate and uridylic and guanylic acids with 1.0 *M* acetic acid – 0.15 *M* sodium acetate (5). (B) For preparative purposes, a more rapid method for separating fraction X from mononucleotides consisted of equilibrating a 25 cm×3.3 cm column of Dowex-1-acetate with 500 ml of 1.0 *M* acetic acid – 0.15 *M* sodium acetate and then charging the column with the neutralized hydrolyzate which had been made 1.0 *M* with respect to acetic acid and 0.15 *M* with respect to sodium acetate. Elution was continued with 1.0 *M* acetic acid – 0.15 *M* sodium acetate. For 18-hour hydrolyzates, 3 liters of 1.0 *M* acetic acid – 0.15 M sodium acetate was passed through the column after the optical density (260 mμ) of the effluent had decreased to 0.3–0.4 before elution of fraction X was commenced. For 4-hour hydrolyzates, 14 liters of 1.0 *M* acetic acid – 0.15 *M* sodium acetate was passed through the column after the optical density (260 mμ) of the effluent had decreased to about 1.0 before fraction X was eluted. Fraction X was eluted with aqueous solutions of sodium sulphate and acetic acid. Fraction X_1 was eluted by 0.2 *M* sodium sulphate – 0.5 *M* acetic acid, fraction X_2 by 0.6 *M* sodium sulphate – 0.5 *M* acetic acid, and fraction X_3 by 0.6 *M* sodium sulphate – 1.5 *M* acetic acid. This preliminary separation into three subfractions was necessary in order to achieve adequate separations by subsequent chromatography on DEAE-cellulose. The trailing region which followed fraction X_1 and preceded fraction X_2 contained a mixture of compounds found in fractions X_1 and X_2. It is likely that the dinucleotide ApCp would be eluted prior to the elution of fraction X. Figures 1 and 2 show the fractional elution of the postmononucleotide fractions of 18- and 4-hour hydrolyzates in 1 *M* potassium hydroxide respectively. The yields of postmononucleotide fraction were 4.5% and 22% for the 18- and 4-hour hydrolyzates of commercial preparations of RNA respectively.

(*iii*) *Chromatographic separation of the constituents of fractions X_1, X_2, and X_3 on DEAE-cellulose columns.*—The use of DEAE–cellulose columns permitted the separations on a large scale to be carried out at pH levels near neutrality and thus avoided hydrolysis of glycosidic or internucleotide linkages. The DEAE-cellulose was prepared for chromatography by batchwise equilibration of 2 g of commercial DEAE-cellulose with 40 ml of buffer which was molar with respect to the salt of the anion being used. The equilibration with molar buffer for 2 or 3 minutes was repeated four times with fresh buffer. The DEAE-cellulose was then equilibrated batchwise five times with a 20-fold dilution of the same buffer (i.e. 0.05 *M* with respect to the salt of the anion being used) and finally suspended in the latter buffer and packed in a column under a pressure of 5 p.s.i. This provided a 25 cm × 1.2 cm column. Thus if a chromatogram was to be developed at pH 5.4, the DEAE-cellulose was equilibrated five times with fresh 1.0 *M* sodium acetate, pH 5.4 (1.0 *M* sodium acetate, 0.175 *M* acetic acid), and then five more times with 0.05 *M* sodium acetate,

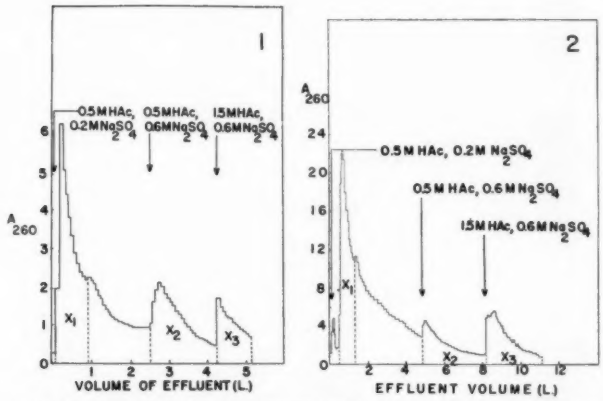


FIG. 1. The fractional elution of oligonucleotides from a 25 cm × 3.3 cm column of Dowex-1-acetate. The oligonucleotides were derived from an 18-hour hydrolyzate of 10 g of commercial RNA.

FIG. 2. The fractional elution of oligonucleotides from a 25 cm × 3.3 cm column of Dowex-1-acetate. The oligonucleotides were derived from a 4-hour hydrolyzate of 10 g of commercial RNA.

pH 5.4. Next the DEAE-cellulose was suspended in 0.05 *M* sodium acetate, pH 5.4, and packed in a column. The column was charged with a solution of the compounds to be chromatographed which was made 0.05 *M* with respect to sodium acetate at pH 5.4. Mononucleotides and dinucleotides were held on the column under the conditions given above provided that the charging solution was salt-free before it was made 0.05 *M* with respect to sodium acetate. The flow rates were not critical but were maintained at 50–60 ml per hour for all columns. The most useful systems have been DEAE-acetate at pH 5.4 and pH 4.6. Figures 3, 4, and 5 show the chromatography of fractions X_1, X_2, and X_3 respectively on 25 cm × 1.2 cm DEAE-acetate columns. Recoveries from the DEAE–cellulose columns have always been 95–100%. The peaks were chromatographed in solvent (*a*) to obtain information about their degree of purity. The lines of division between the peaks indicate the boundaries of the fractions which were individually collected, desalted, and chromatographed on paper. The ultraviolet photograph of the paper chromatogram corresponding to the column chromatogram of Fig. 4 is shown in Fig. 6. Figure 7 shows the results of chromatographing a neutralized 2-hour alkali hydrolyzate of yeast RNA (11) using solvent (*a*). The compounds which remain to be identified have been designated by the symbol D and an appropriate subscript. It is evident from the figures that GpCp and ApUp were found in the same peak and hence further separation was needed. A column fractionation could be achieved by passing the mixture in 0.175 *M* acetic acid through a Dowex-50 column (in the H^+ form) which was equilibrated with 0.175 *M* acetic acid. The ApUp, by virtue of its high negative charge, passed through directly whereas the GpCp took a much longer time to be eluted and could be brought off as a fairly sharp peak with 1.0 *M* sodium acetate, pH 5.4.

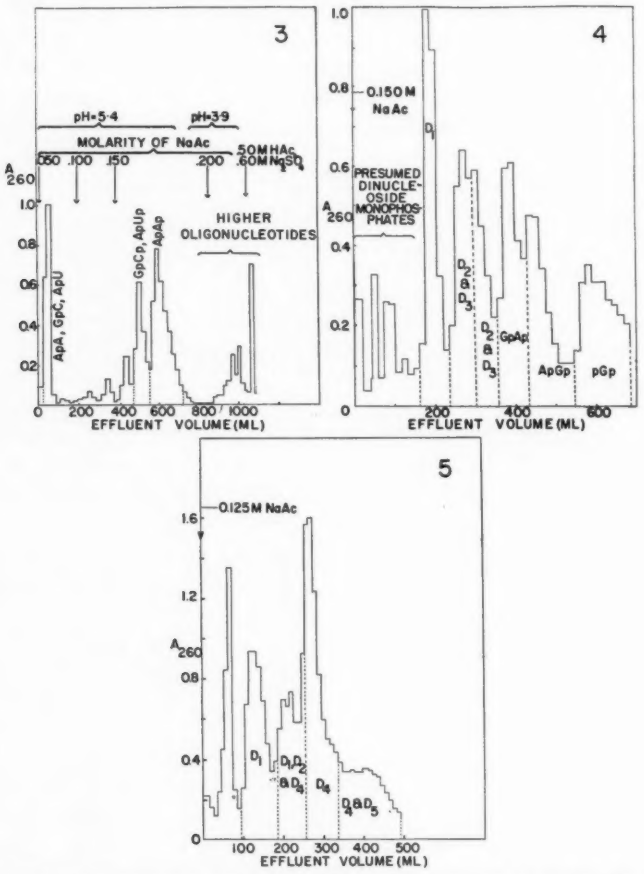


FIG. 3. The chromatography of fraction X_1 on DEAE-acetate at pH 5.4. The fraction was derived from an 18-hour digest of calf liver RNA.

FIG. 4. The chromatography of fraction X_2 on DEAE-acetate at pH 5.4. The fraction was derived from an 18-hour digest of commercial RNA.

FIG. 5. The chromatography of fraction X_3 on DEAE-acetate at pH 4.6. The fraction was derived from an 18-hour digest of commercial RNA. D_5 is now known to be GpGp.

Preparative Procedures for Dinucleotides

The following procedures were used for chromatographic separation of the dinucleotides of fractions X_1, X_2, and of the region between these fractions.

(*i*) *Preparation of ApAp from an 18-hour hydrolyzate of commercial RNA.*— Fraction X_1, in 0.05 *M* sodium acetate, pH 4.6, was chromatographed on a 25 cm × 2.2 cm DEAE-acetate column which had been equilibrated with 0.05 *M* sodium acetate, pH 4.6. Elution was carried out with 0.100 *M* sodium acetate, pH 4.6. The chromatogram is shown in Fig. 8. The fraction bounded by the dashed lines was adsorbed on charcoal, eluted, and the eluate evaporated to dryness. The dry residue was dissolved in water and the solution lyophilized

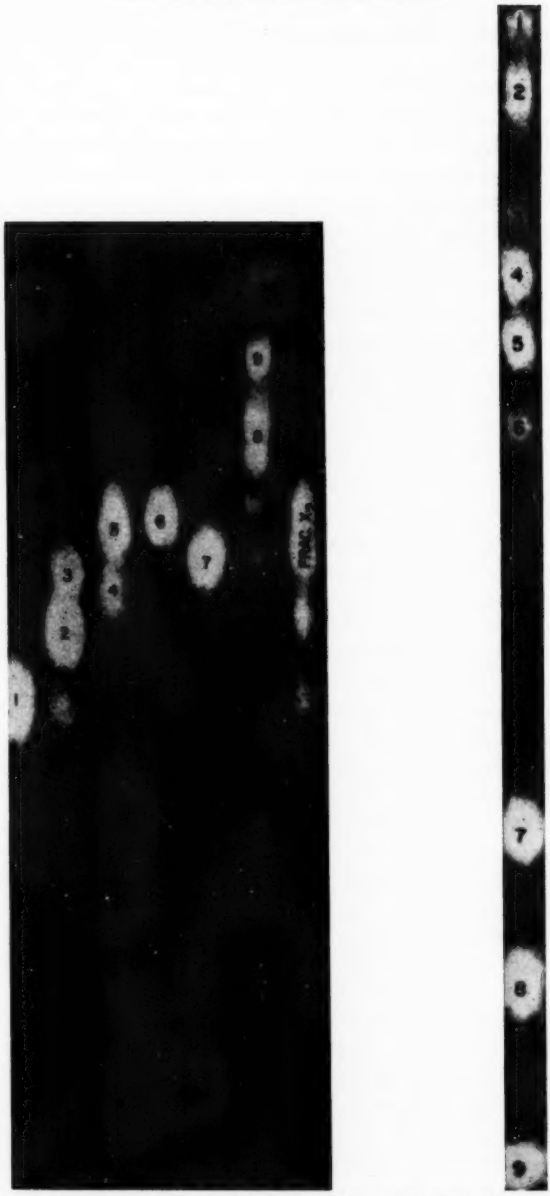


FIG. 6 (left). The chromatography of the fractions indicated in Fig. 4 on Whatman No. 3 paper using solvent (*a*). The spots are: 1—D_1; 2—D_2; 3—D_3; 4—ApAp; 5—GpAp; 6—ApGp; 7—pGp; 8, 9—oligonucleotides eluted with 0.6 *M* Na_2SO_4, 0.5 *M* HAc from DEAE-acetate but not shown in Fig. 4.

FIG. 7 (right). The chromatography of a 2-hour hydrolyzate of yeast RNA (11) on Whatman No. 3 paper using solvent (*a*). The spots are: 1—tetranucleotides; 2—trinucleotides; 3—GpGp; 4—ApGp, GpAp, GpCp; 5—ApAp, ApCp, GpUp; 6—ApUp; 7—Gp; 8—Ap, Cp; 9—Up.

to give a white powder. The material was pure both by analysis and by paper chromatography in solvent (*a*). This procedure yielded about 40 mg of ApAp from 10 g of commercial RNA.

(*ii*) *Preparation of ApAp and ApUp from a 4-hour hydrolyzate of commercial RNA.*—Fraction X_1, in 0.05 *M* sodium acetate, pH 4.6, was chromatographed on a 25 cm × 2.2 cm DEAE-acetate column which had been equilibrated with 0.05 *M* sodium acetate, pH 4.6. Elution was carried out with 0.125 *M* sodium acetate, pH 4.6. The chromatogram is shown in Fig. 9. The first peak contained mainly ApUp and small amounts of GpCp. The GpCp could be removed with Dowex-50 as described earlier. The second peak contained ApAp and small amounts of ApGp and GpAp. The ApAp could be separated from GpAp and ApGp by the following method. Commercial DEAE-cellulose was suspended in 0.05 *M* sodium chloride and packed in a column. The mixture of ApAp, GpAp, and ApGp was made 0.05 *M* with respect to sodium chloride and adsorbed on the column. The elution was carried out with 0.100 *M* sodium chloride. The first peak obtained contained ApAp and the GpAp and ApGp came off subsequently. The ApAp was analytically and chromatographically pure. This method yielded about 30 mg of ApUp and 25 mg of ApAp from 10 g of commercial RNA.

(*iii*) *Preparation of ApGp and GpAp from a 4-hour hydrolyzate of commercial RNA.*—The trailing region bounded by the dashed lines between fractions X_1 and X_2 was used to prepare GpAp and ApGp. The fraction, in 0.05 *M* sodium acetate, pH 4.6 was adsorbed on a 25 cm × 1.2 cm column of DEAE-acetate equilibrated with 0.05 *M* sodium acetate, pH 4.6. Elution was carried out with 0.100 *M* sodium acetate, pH 4.6. An exemplary chromatogram is shown in Fig. 10. The method yielded about 40 mg of GpAp and 30 mg of ApGp from 10 g of commercial RNA. Small amounts of ApAp present in the GpAp could be removed by chromatography on a DEAE-cellulose column eluted with 0.100 *M* sodium chloride.

(*iv*) *Preparation of GpCp from a 4-hour hydrolyzate of commercial RNA.*—The small peak preceding fraction X_1, in 0.05 *M* sodium acetate, pH 4.6, was chromatographed on a 25 cm × 1.2 cm DEAE-acetate column equilibrated with 0.05 *M* sodium acetate, pH 4.6. Elution was effected with 0.125 *M* sodium acetate, pH 4.6. The chromatogram is shown in Fig. 11. This method yielded about 15 mg of GpCp from 10 g of commercial RNA.

(*v*) *Preparation of GpCp from a 4-hour hydrolyzate of calf liver RNA isolated by the method of Pain and Butler.*—Fraction X_1 in 0.05 *M* ammonium chloride, pH 6.8 was chromatographed on a 25 cm × 1.2 cm DEAE-chloride column equilibrated with 0.05 *M* ammonium chloride, pH 6.8. The first peak off the column contained GpCp which was cleanly separated from subsequent peaks. This method yielded about 125 mg of GpCp from 10 g of RNA.

The following methods were used to prepare the dinucleotides UpUp and CpCp which were not found in alkali hydrolyzates of RNA.

(*i*) *Preparation of diuridylic acid from a 30-minute alkali hydrolyzate of polyuridylate.*—Polyuridylate was prepared in the same manner as previously described for polycytidylate. A measurement of the time course of

hydrolysis of polyuridylate in 0.86 *M* potassium hydroxide at 26° C showed that the yield of diuridylic acid reached a maximum at 30 minutes. A 30-minute hydrolyzate of 6 mg of polyuridylate was neutralized with 72% perchloric acid and stored at 0° C for 3 hours (in order to reduce the concentration of potassium perchlorate to 0.06 *M*) before charging on a 25 cm × 1.2 cm DEAE-acetate column pre-equilibrated with 0.05 *M* sodium acetate, pH 5.4. The elution of products was carried out as indicated in Fig. 12. The yields of uridylic acid (36%), diuridylic acid (29%), triuridylic acid (19%), and higher oligonucleotides (16%) obtained from DEAE-cellulose columns were verified and the purity of the first three established by paper chromatography using solvent (*a*).

(*ii*) *Preparation of dicytidylic acid from digests of polycytidylate with pancreatic ribonuclease.*—Alkali hydrolyzates of polycytidylate failed to give more than 10% yields of dicytidylic acid. In order to obtain CpCp, the method of Heppel *et al.* was used (29). It was found that the polycytidylate contained a contaminating nuclease and was completely degraded to cyclic cytidylic acid and cytidylic acid when incubated at pH 7.2 for 1 hour at 37° C. Two treatments of the polycytidylate with phenol by the method of Kirby (30) destroyed the nuclease activity. Ten milliliters of polycytidylate (1.2 μmoles of constituent mononucleotide per ml) were mixed with 0.1 ml of 1.1 *M* potassium phosphate buffer, pH 7.2, and 0.1 ml of pancreatic RNase (0.02 mg Worthington RNase/ml) and incubated at 37° C for 1 hour. The digest was brought to pH 1.2 by the addition of 0.1 ml of 12 *M* hydrochloric acid and stored for 3 hours at 23° C before bringing to pH 5.0 by the addition of 0.1 ml of 9 *M* potassium hydroxide. The digest containing the mixture of oligonucleotides was adsorbed on charcoal, eluted with ammoniacal ethanol, evaporated, and dissolved in 10 ml of water. The desalted digest was chromatographed on DEAE-acetate at pH 5.4 and gave the same type of elution pattern shown in Fig. 12 except that the dicytidylic acid came off in two peaks. The compounds in the two peaks were identical by paper chromatography in solvents (*a*) and (*b*) and had the expected R_f values. The compounds had a slightly smaller R_f value than ApAp in solvent (*a*) and the same R_f as UpUp in solvent (*b*). It was presumed by analogy with the order of elution of the 2′- and 3′-mononucleotides from Dowex-1, and with the data given by Merrifield and Woolley (31) for the elution of CpC2p and CpC3p from Dowex-1, that the first peak

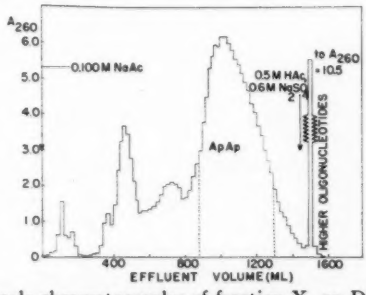


FIG. 8. The large-scale chromatography of fraction X_1 on DEAE-acetate at pH 4.6. The fraction was derived from an 18-hour digest of commercial RNA.

contained CpC2p and the second peak contained CpC3p. It is interesting to note that there were 1.7 moles of CpC2p per mole of CpC3p, which is in accord with the fact that Merrifield and Woolley obtained twice as much CpC2p as CpC3p in their *acid* hydrolyzates of RNA. This finding is in contrast to the preponderance of the 3′-isomer obtained by *alkali* hydrolysis. The yield of dicytidylic acid from the RNase digest was 44%.

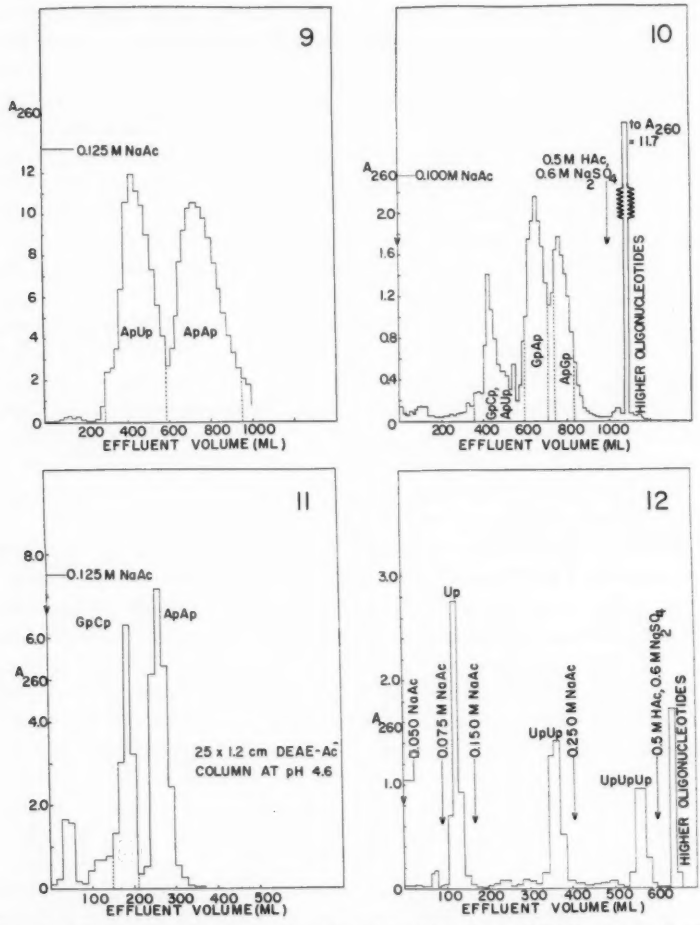


FIG. 9. The large-scale chromatography of fraction X_1 on DEAE-acetate at pH 4.6. The fraction was derived from a 4-hour digest of commercial RNA.

FIG. 10. The separation of GpAp and ApGp on DEAE-acetate at pH 4.6. The fraction used for chromatography was derived from a 4-hour digest of commercial RNA. See text for details.

FIG. 11. The large-scale separation of GpCp and ApAp on DEAE-acetate at pH 4.6. The fraction was derived from a 4-hour digest of commercial RNA. See text for details.

FIG. 12. The elution from DEAE-acetate of the products formed by hydrolyzing polyuridylate in 0.86 *M* KOH for 30 minutes at 26° C.

Results and Discussion

Characterization of Dinucleotides

The dinucleotides isolated from alkali hydrolyzates of RNA were identified by the following procedures.

(*a*) Dinucleotide —Prostatic Phosphomonoesterase→ Dinucleoside monophosphate —1 *M* KOH, 24 hr, 26° C→ Nucleoside of P-terminal residue, 2′- and 3′-nucleotides of S-terminal residue

(*b*) Dinucleotide —Rye-grass Nuclease, pH 5.4→ 2′- and 3′-Nucleotides of P-terminal residue, nucleoside cyclic phosphate of S-terminal residue

(*c*) Dinucleotide —Prostatic Phosphomonoesterase→ Dinucleoside monophosphate —Venom diesterase→ 5′-Nucleotide of P-terminal residue, nucleoside of S-terminal residue

(*d*) Dinucleotide —Venom Phosphodiesterase→ Nucleoside 2′,5′-diphosphate and nucleoside 3′,5′-diphosphate of P-terminal residue, nucleoside of S-terminal residue

(*e*) Dinucleotide —Pancreatic Ribonuclease→ 2′- and 3′-Nucleotides of P-terminal residue, nucleoside cyclic phosphate of S-terminal residue

The method of Whitfeld (32) was applied to ApAp and gave only the 3′-isomer of adenylic acid. This evidence, in conjunction with the results of methods (*c*) and (*d*), shows that the internucleotide linkage is from the 3′-position of the S-terminal group to the 5′-position of the P-terminal group.

The members of the pairs of products formed by procedures (*a*), (*c*), (*d*), and (*e*) were formed in equimolar amounts but the products formed by procedure (*b*) were not because of contaminating nucleotidase activities. The qualitative results obtained by method (*b*) were always definite and in agreement with the results of the other four methods. The results obtained by applying the different procedures to five dinucleotides are tabulated in Table I. The evidence for the identification of guanosine 2′,5′-diphosphate and/or 3′-5′-diphosphate is that its only base is guanine, it is dephosphorylated by prostatic phosphomono-

TABLE I

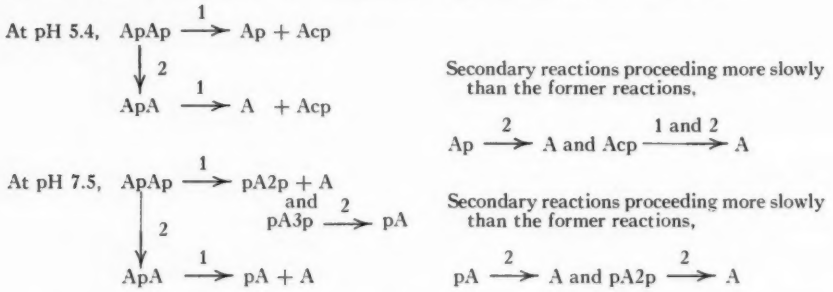
Evidence for the structure of the dinucleotides isolated from alkali hydrolyzates of RNA

Dinucleotide	Products formed by procedures (see text)				
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)
ApAp	A,A2p,A3p	A2p,A3p,Acp	pA,A	pA2p,pA3p,A	A2p,A3p,Acp
ApGp	G,A2p,A3p	G2p,G3p,Acp	pG,A	pGp,A	Trace hydrolysis
GpAp	A,G2p,G3p	A2p,A3p,Gcp	pA,G	pA2p,pA3p,G	Trace hydrolysis
ApUp	U,A2p,A3p	Up,Acp	pU,A	pUp,A	Up,A3p,Acp
GpCp	C,G2p,G3p	Cp,Gcp	pC,G	pCp,G	

NOTE: It is noteworthy that procedure (*b*) yields both of the possible nucleosides because of the contaminant nucleotidase activity of the rye-grass preparation. The dinucleotide GpGp was identified by procedure (*a*). The compound indicated by the symbol D_5 in Fig. 5 is now known to be GpGp.

esterase to guanosine and it has the same R_f as authentic guanosine 2′,/3′,5′-diphosphate in solvents (*a*), (*b*), (*c*), and (*d*). Authentic guanosine 2′,/3′,5′-diphosphate was obtained by treating ApGp with purified venom phosphodiesterase. Guanosine 2′,/3′,5′-diphosphate has not been detected in alkali hydrolyzates of liver RNA.

Results obtained by digesting ApAp with rye-grass nuclease are given in Table II. It may be seen that the activity of the enzyme preparation depends very markedly on the pH. At pH 5.4, the products of digestion were A2p, A3p, Acp, A, and ApA. At pH 7.5, the products of digestion were pA, pA2p, A, and ApA. These activities may be briefly summarized as follows.



The numeral "1" denotes a phosphodiesterase activity and the numeral "2" denotes a phosphomonoesterase activity.

Although treatment with oxalate inactivated much of the nucleotidase activity in the digests at pH 5.4, a substantial dephosphorylation of nucleotides still occurred. No attempt was made to inactivate the nucleotidase activity in digests at pH 7.5. The optimal pH for the nucleotidase activity was 7.5 and 3′-phosphate groups were removed much more quickly than were 2′- or 5′-groups. Thus, by carefully regulating the concentration of enzymes, it was possible to obtain complete hydrolysis of 3′-phosphate groups (from ApA3p and pA3p) without having hydrolysis of 2′- or 5′-groups. It was presumed that no hydrolysis of 2′- and 5′-phosphoryl groups had occurred in the digest at pH 7.5 since pA and A2p suffered no hydrolysis in control experiments under the same conditions. Furthermore the sum of the number of moles of pA and pA2p was equal to the number of moles of A (see Table II) as would have been expected if no hydrolysis of 2′- and 5′-phosphate groups had occurred. The amount of pA2p in such a digest gave a measure of the amount of ApA2p before hydrolysis and the sum of the amounts of ApA and pA gave a measure of the amount of ApA3p before hydrolysis. From the results of the digestion at pH 7.5 given in Table II, it can be seen that 34% of the singly esterified phosphate in the sample of diadenylic acid was 2′ and 66% was 3′. Chromatographic identification of the products formed at pH 7.5 was made in solvents (*a*), (*b*), (*c*), and (*d*), using authentic samples of A2p, A3p, pA2p, pA3p, pA, A, and ApA as standard markers. Solvents (*b*) and (*c*) were particularly useful here since the order of migration of pA2p, pA, and ApA in solvent (*b*) was reversed in solvent (*c*). It is noteworthy that the dephosphorylation of pA3p at pH 7.5

must be complete since we were unable to obtain a clean-cut paper chromatographic separation of pA2p and pA3p. Thus, we have been unable to estimate the proportion of 2′- and 3′-terminal phosphates of ApAp by degradation with snake venom phosphodiesterase and estimation of the relative amounts of pA2p and pA3p formed. Semi-quantitative analysis has indicated a substantial excess of pA3p compared with pA2p in such digests. In solvent (*a*) it was found that authentic pA2p had the same R_f as ApUp (see Fig. 7) and pA3p moved just ahead of pA2p but did not separate from it completely.

TABLE II

Products formed by hydrolysis of ApAp with enzymes from rye grass

Product	Digestion with "x" units of enzyme preparation at pH 5.4	Digestion with "$8x$" units of enzyme preparation at pH 5.4	Digestion with "$0.7x$" units of enzyme preparation at pH 7.5
ApAp	20.6%	0.0%	0.0%
ApA	15.5	0.0	38.5
Ap	30.7	34.2	0.0
Acp	25.8	40.0	0.0
pA	0.0	0.0	13.5
pA2p	0.0	0.0	17.2
A	7.4	25.8	30.8
	100.0%	100.0%	100.0%

NOTE: The figures in Table II represent the percentages of the total ultraviolet absorption of the digestion products at 260 mμ, and these percentages are taken to represent the molar distribution of adenosine among the digestion products. Differences in the molar extinction coefficient of adenosine in its various constituent forms are considered negligible.

From the digestion at pH 7.5, % ApA2p = (2×17.2)/100 = 34%.
% ApA3p = (2×13.5+38.5)/100 = 66%.

Hydrolyses were carried out for 2 hours at 37° C. The substrate concentration was 0.50 μmole/ml of digest in all cases. The concentration of the enzyme preparation has been expressed in terms of "x" where x = 0.057 mg of enzyme preparation/ml of digest. The digests at pH 5.4 were buffered in 0.3 *M* NaAc, pH 5.4, and analyzed by quantitative paper chromatography using solvent (*d*). The digest at pH 7.5 was buffered in 0.01 *M* TRIS, pH 7.5, and analyzed by quantitative paper chromatography using solvent (*c*).

Snake venom phosphodiesterase caused complete hydrolysis of ApA in 2 hours whereas only 15% of ApAp was hydrolyzed under the same conditions. ApGp and GpAp were degraded to the extent of 60% after 24 hours whereas ApAp was degraded to the extent of 95% in the same period.

Pancreatic ribonuclease caused only traces of hydrolysis of GpAp and ApGp after 24 hours. Twenty-five per cent of ApUp was hydrolyzed in 24 hours. Sixty per cent hydrolysis of ApAp was achieved in 24 hours but ApA was unaffected under the same conditions indicating perhaps a cationic site on the enzyme which is involved in interaction with substrate. Extending the incubation time to 72 hours caused complete hydrolysis of ApAp to Acp and Ap. The concentration of enzyme required to carry out these hydrolyses is very great, being roughly equivalent to the substrate concentration on a dry weight basis. It has not been established whether the hydrolysis is effected by ribonuclease A or B (or perhaps by both), or if it is caused by a trace contaminant of RNase which only has an effect by virtue of the large concentration of enzyme employed. The formation of the cyclic nucleotide from the S-terminal group and the stability of Acp to further degradation are, however, consistent with the hypothesis that the hydrolysis of ApAp is effected by RNase.

Column chromatography of alkali hydrolyzates of ApAp and ApUp on Dowex-1-acetate have yielded the results expected from the paper chromatographic data. ApAp yielded A2p and A3p with a substantial excess of the latter. ApUp yielded A2p, A3p, and Up.

Studies on the alkali-stable compounds (D_1, D_2, D_3, and D_4) have indicated that procedures (*a*) and (*b*) convert them to compounds with the paper chromatographic properties of dinucleoside monophosphates. The failure of rye-grass nuclease to cause degradation of the dinucleoside monophosphates is strong auxiliary evidence (in addition to stability in alkali) that the 2′-position of the S-terminal group is substituted, and as a result a cyclic phosphate intermediate cannot form. The S-terminal group is not deoxyribose since a negative reaction was obtained in a diphenylamine test.

Action of Alkali on Dinucleotides

The proportions of 2′- and 3′-isomers formed from the S-terminal residue when dinucleotides were hydrolyzed in alkali are shown in Table III. The

TABLE III

The proportions of 2′- and 3′-isomers formed from alkali hydrolysis of dinucleotides and the corresponding dinucleoside phosphates

Compound	Percentages produced			
	A3p	G3p	A2p	G2p
ApAp	66%		34%	
ApA	50%		50%	
ApGp	58%		42%	
ApG	60%		40%	
GpAp		55%		45%
GpA		58%		42%
GpCp		64%		36%
GpC		61%		39%

effect of removing the singly esterified phosphate from ApGp, GpAp, and GpCp was shown to be insignificant but there appears to be a significant change in the relative amounts of the 2′- and 3′-isomers formed when the singly esterified phosphate is removed from ApAp. The change caused by removal of the singly esterified phosphate group appears to be significant from the following information. When 1 mole of diadenylic acid was hydrolyzed in alkali, the 2 moles of phosphate were found in the 0.68 mole of A2p and the 1.32 moles of A3p formed. For the same sample of diadenylic acid, it was found that 0.34 mole of the singly esterified phosphate per mole of diadenylic acid was on the 2′-position, and 0.66 mole was on the 3′-position (using the rye-grass preparation at pH 7.5). Thus the proportions of isomers formed from the S-terminal group of diadenylic acid during hydrolysis in alkali were 0.34 mole of A2p and 0.66 mole of A3p.

Since the isomers were formed in equal amounts when ApA was hydrolyzed in alkali, it would appear that the presence of the terminal phosphate produced a bias which favored formation of the 3′-phosphate from the S-terminal residue. All analyses for 2′- and 3′-isomers were performed with solvent (*b*),

and in the case of ApA hydrolyzates it was necessary to estimate the amounts of A and A3p by calculation since A and A3p do not separate cleanly in this solvent. That is, A + A3p = measurable quantity, and A − A3p = A2p = measurable quantity and hence A and A3p can be determined by solving these equations. The reason why removal of the terminal phosphate had no effect on the proportion of 2′- and 3′-isomers formed from ApGp, GpAp, and GpCp but had a marked effect on the proportion of these isomers formed from ApAp may be related to the fact that ApAp was the only one of the dinucleotides which had the same base in both the S- and P-terminal residues.

Studies on the time courses of the alkali hydrolysis of dinucleotides showed that the hydrolyses were first order reactions for fixed concentration of alkali and the concentration of dinucleotide used (0.5 μmole of dinucleotide per ml of hydrolyzate). Samples from the digests were neutralized at different times with 3 *M* perchloric acid and subsequently chromatographed on paper in solvent (*a*). The spots were eluted with water after chromatography, and the percentage of mononucleotide was estimated as the percentage of ultraviolet absorption at 260 mμ (no correction was made for the increase in absorption occurring as the result of hydrolysis). The first order rate constants are given in Table IV. The widely differing rates of hydrolysis of internucleotide linkages are evident from the fact that UpUp hydrolyzed 10 times faster than ApAp. The relative magnitudes of the rate constants explain why (i) ApAp was always the major non-mononucleotide component of our alkali hydrolyzates, (ii) the non-mononucleotide fraction of alkali hydrolyzates was predominantly purine in its base composition, (iii) GpCp and ApUp were found in smaller amounts and sometimes not at all in 24-hour hydrolyzates whereas ApAp, ApGp, and GpAp were always found.

TABLE IV

First order rate constants for the hydrolysis of dinucleotides in 0.86 *M* potassium hydroxide at 26° C

Dinucleotide	Rate constant
UpUp	1.0
CpC3p	0.62
CpC2p	0.38
GpCp	0.25
ApUp	0.25
ApGp	0.21
GpAp	0.18
ApAp	0.10

NOTE: The constants are estimated to be reliable within ±5%.

The variability in the rates of alkali hydrolysis of various fractions of yeast and pancreas RNA isolated by electrophoretic convection has been attributed to the occurrence of different proportions of internucleotide linkages with different rates of hydrolysis (33). The relation between the rate of hydrolysis of the internucleotide linkage of a dinucleotide (say ApAp) and the rate of hydrolysis of the same linkage when it is found in a polymer (say UpGpCpAp-GpUp*A*p*A*pGpUpCp) has not been investigated but it might reasonably be

supposed that an internucleotide linkage such as occurs in ApAp would be more stable to hydrolysis than an internucleotide linkage such as that in UpUp even when these linkages occur in a polymer. A further implication might be that there is a rapid initial attack at the weaker linkages by alkali, and that the occurrence of a series of adjacent purine nucleotides in a particular type of ribonucleic acid would confer on it a relatively greater resistance to degradation by alkali than would be exhibited by a type of ribonucleic acid lacking such sequences.

The dependence of the rate of hydrolysis of ApAp on concentration of potassium hydroxide is shown in Fig. 13. The first order rate constants were calculated from data obtained by quantitative chromatography using solvent (*b*).

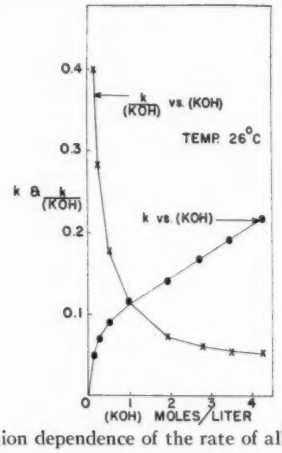


FIG. 13. The concentration dependence of the rate of alkali hydrolysis of ApAp.

The first order rate constant for the hydrolysis of ApAp in 1 *M* sodium hydroxide was the same as it was for hydrolysis in 1 *M* potassium hydroxide at 26° C. When ApAp was dephosphorylated and purified by charcoal adsorption, it was found that the first order rate constant in 1.0 *M* potassium hydroxide for ApA was 0.140 as compared with 0.115 for ApAp in 1.0 *M* potassium hydroxide.

The rate constants obtained for CpC2p and CpC3p (Table IV) must be considered as tentative since the separation on DEAE-acetate was not complete and also because there were insufficient amounts of the materials available to allow the experiments to be repeated. However, the apparent difference in the rates of hydrolysis of the isomers may be attributable to the fact that the 2′-hydroxyl group of the P-terminal residue is dissociated under the alkaline conditions used (34), and this group may, by virtue of the additional negative charge in CpC3p, accelerate the effect of the negatively charged hydroxyl ions in catalyzing hydrolysis of the internucleotide linkages. The increased rate of hydrolysis of ApA relative to the rate of hydrolysis of ApAp could also be the result of the liberation of more 2′-hydroxyl groups from the P-terminal residues of ApA2p. The tentative results obtained with dicytidylic acid emphasize

that the rate constants given for the other dinucleotides must be considered as weighted averages since the dinucleotides were mixtures in which roughly 60% of the singly esterified phosphate groups were 3′ and 40% were 2′.

TABLE V

Quantitative data for a 2-hour hydrolyzate of yeast RNA in 0.86 *M* potassium hydroxide at 26° C

Spot number*	Compounds	% of total absorption of hydrolyzate at 260 mμ
1 and 2	Higher oligonucleotides	21.4
3	GpGp	3.8
4	ApGp, GpAp, GpCp	10.0
5	ApAp, ApCp, GpUp	11.8
6	ApUp	5.1
7	Gp	13.9
8	Ap, Cp	18.2
9	Up	15.8

*cf. Fig. 7.

Dinucleotides in a 2-Hour Hydrolyzate of Yeast RNA

The paper chromatography in solvent (*a*) of a 2-hour hydrolyzate of yeast RNA (11) in 0.86 *M* potassium hydroxide at 26° C is shown in Fig. 7. The relative proportion of the total absorption at 260 mμ which is found in each spot is indicated in Table V. It can be seen that about 50% of the hydrolyzate is in the form of mononucleotides, 30% is in the form of dinucleotides, and 20% is in the form of higher oligonucleotides. Chromatography on paper using solvent (*a*) separates the dinucleotides into four groups: (i) GpGp, (ii) ApGp, GpAp, GpCp, (iii) ApAp, ApCp, GpUp, and (iv) ApUp. The GpCp of group (ii) can be separated from the mixture of ApGp and GpAp by paper chromatography in solvent (*b*). The compounds of group (iii) were separated cleanly by paper chromatography using solvent (*b*). The dinucleotides were identified by method (*a*) except for ApGp and GpAp which could not be separated by paper chromatography. The mixture of ApGp and GpAp gave the expected results using method (*a*) and there is no doubt of their identity since they have been repeatedly identified in our alkali hydrolyzates after separation on DEAE-cellulose.

Column chromatography of the 2-hour hydrolyzate on DEAE-acetate at pH 5.4 confirmed the relative proportions of mononucleotides, dinucleotides, and higher oligonucleotides found by paper chromatography. Mononucleotides were eluted with 0.075 *M* sodium acetate. Two sharp, well-separated peaks were obtained, the first containing the pyrimidine mononucleotides and the second containing the purine mononucleotides. The dinucleotides were eluted in three peaks using 0.150 *M* sodium acetate. The first peak was relatively small, the second peak contained ApCp, ApUp, GpCp, and GpUp while the third peak which was quite broad contained ApAp, GpAp, ApGp, and GpGp. It was found that the UpUp and CpCp detected by spotting the hydrolyzate on paper chromatograms at high concentrations were found in the first small peak eluted by 0.150 *M* sodium acetate. The small peak represented about 10% of the dinucleotides found in the hydrolyzate. The compounds of the

third peak were subjected to rechromatography on DEAE-acetate at pH 4.6 and were eluted in three well-separated peaks. The first peak contained ApAp and GpAp, the second ApGp, and the third GpGp.

Each of the eight possible diribonucleotides which are not susceptible to pancreatic ribonuclease (ApAp, ApGp, ApCp, ApUp, GpGp, GpAp, GpCp, and GpUp) is present in an amount varying between 3 and 5% of the total 2-hour hydrolyzate of yeast RNA whereas the eight possible diribonucleotides which are susceptible to pancreatic ribonuclease (CpCp, CpUp, CpAp, CpGp UpUp, UpCp, UpAp, and UpGp) are present in much smaller amounts.

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EVALUATION OF PROTEIN IN FOODS

III. A STUDY OF BACTERIOLOGICAL METHODS[1]

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Abstract

Bacteriological methods for the determination of protein quality were evaluated by comparison with protein efficiency ratio (P.E.R.) values determined by a standardized rat growth assay. Enzyme or acid hydrolyzates of foods were used as the source of amino acids with hydrolyzed whole egg powder as the reference standard. With *Streptococcus faecalis* A.T.C.C. 9790 autolysis occurred in media containing hydrolyzates of proteins deficient in lysine, and was largely responsible for results which did not agree with P.E.R. values. In methods employing *Leuconostoc mesenteroides* P-60 A.T.C.C. 8042, growth was influenced only by the most limiting amino acid relative to the requirements of the test organism.

Results with enzyme hydrolyzates correlated poorly with P.E.R. values, whereas, with acid hydrolyzates, a good correlation was obtained for cereal proteins. A difference in amino acid requirements was largely responsible for the lack of agreement between the P.E.R. assay and methods employing *L. mesenteroides*, particularly for legumes and foods of animal origin. It was concluded that bacteriological assay methods which have been proposed for protein evaluation are unsatisfactory as screening procedures for the evaluation of protein in foods.

Introduction

Current emphasis on the importance of protein in the diet has pointed to the need for a simple, rapid, and inexpensive method of protein evaluation. In recent years several methods were proposed (1–6) employing ciliated protozoa of the genus *Tetrahymena*. Although they offered the advantage that the samples did not require hydrolysis prior to assay, good correlation with the results of animal assays was reported for only a limited number of protein sources. Halevy and Grossowicz (7), using a strain of *S. faecalis*, measured the nutritive value of egg albumin, gelatin, gluten, and zein in relation to casein following in vitro digestion of the proteins with a crude preparation of pancreatic proteinases. A simple method of protein evaluation was also described by Teeri, Virchow, and Loughlin (8) with *S. faecalis* A.T.C.C. 9790 in a medium in which amino acids were supplied only by enzyme hydrolyzates of proteins. *L. mesenteroides* P-60 was used by Horn, Blum, and Womack (9) to determine the effect of heat treatment on the nutritive value of cottonseed proteins after enzyme and acid hydrolysis. In these methods it was assumed that the growth of the test organism was dependent upon the pattern of amino acids, available in the protein hydrolyzate, and although results agreed, for a limited number of proteins, with literature values determined by animal assay, it was not shown that they could be used as general procedures to evaluate the proteins in natural foods.

This paper describes the results of an investigation of bacteriological methods for the measurement of nutritive value of protein in a variety of foods of both plant and animal origin. The methods were evaluated by comparison

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with protein efficiency ratio (P.E.R.) determined by a standardized rat growth assay.

Materials and Methods

Culture Maintenance

The AOAC method (10) for the assay of folic acid was used for maintenance of stock cultures and preparation of inocula in studies with *S. faecalis* A.T.C.C. No. 9790. Cultures of *L. mesenteroides* P-60 A.T.C.C. No. 8042 were maintained and inocula prepared as outlined in the U.S.P. XV (11) method for the microbiological assay of vitamin B_{12}.

Hydrolysis of Proteins

Enzyme hydrolyzates of foods were prepared by a modification of the procedure of Teeri *et al.* (8) as follows: a sample containing 0.5 g of protein was digested for 24 hours at 37° C with 60 ml of 1.5% pepsin[2] solution at pH 1.2 and then buffered with 10 ml of 30% K_2HPO_4 and the pH adjusted to 8.4 with dilute NaOH. After the addition of 60 mg pancreatin[3] (3 × U.S.P. potency), 20 mg trypsin[4] (1:250), and 20 mg erepsin[4], the digests were layered with toluene and incubated 72 hours at 37° C with constant, gentle agitation. After steaming, to remove the toluene, the hydrolyzates were clarified by filtration, adjusted to pH 6.8, brought to volume, and stored at 6° C under toluene until used. In experiments with *S. faecalis*, hydrolyzates were assayed at levels of 0.10 and 0.30 mg protein per ml. With *L. mesenteroides*, hydrolyzates were assayed at levels of 0.08, 0.12, and 0.16 mg protein per ml.

Acid hydrolyzates of protein foods were prepared by the method of Horn and Blum (13) for the assay of cystine in foods, by reflux of a sample containing 0.5 g protein for 2 hours with 60 ml of 20% HCl. After filtration at pH 4.0, the hydrolyzates were adjusted to pH 7.0 and stored under toluene at 6° C until used.

In assays with acid or enzyme hydrolyzates, cultures were incubated for 72 hours at 33° C. Growth was then measured by titration with 0.1 *N* NaOH to pH 7.0 with a Beckman Model G pH meter.

The extent of digestion of protein in foods after enzyme or acid hydrolysis was determined by the formol titration method of Melnick and Oser (14).

The method of Halevy and Grossowicz (7) was used to determine the order in which amino acids were limiting for growth of the test organisms in the hydrolyzates.

Lysine, cystine, and methionine were estimated in the acid hydrolyzates of foods by microbiological assay employing *L. mesenteroides* with hydrolyzed whole egg powder as the reference standard. Lysine and methionine were determined by the method of Steele *et al.* (15) and cystine by the procedure of Horn and Blum (13).

Protein Efficiency Ratio

The rat growth assay of Chapman, Castillo, and Campbell (16) was used to determine the P.E.R. of each food sample.

[2]Central Scientific Company Inc.
[3]Nutritional Biochemicals Corporation.
[4]Difco Laboratories Incorporated.

Results

Experiments with S. faecalis

Enzyme hydrolyzates of seven protein foods were assayed microbiologically with *S. faecalis* and the results compared with P.E.R. values for the same samples. The bacteriological values were as follows: dried whole egg (standard) 100, oatmeal + milk 130, dried whole milk 111, casein 117, oatmeal 55, "protein cereal" B + milk 131, and "protein cereal" B 48.

The corresponding P.E.R. values, expressed as a percentage of that of egg, were 100, 87, 80, 74, 65, 62, and 6 respectively. The bacteriological values for milk or casein or for cereals with milk were consistently higher than that for the egg standard whereas the value for oatmeal appeared somewhat lower. These results suggested that *S. faecalis* may have a high requirement for lysine. Alternatively, however, it has been reported (17, 18) that autolysis of the cells occurred when *S. faecalis* was grown in synthetic media deficient in lysine. To determine if lysis might account for the lack of agreement between the bacteriological values and P.E.R., *S. faecalis* was grown in media containing enzyme hydrolyzates of dried whole egg, oatmeal, or whole milk powder, with each added at a level that would yield approximately the same amount of growth in 8 hours. Optically matched culture tubes were used and turbidity measurements were made at hourly intervals over an incubation period of 24 hours. The results are shown in Fig. 1. With oatmeal hydrolyzate, lysis

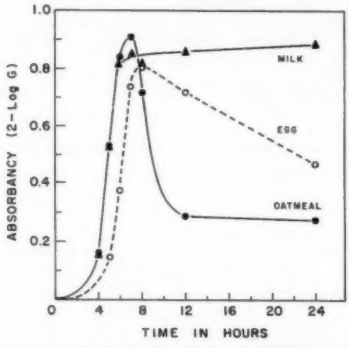

FIG. 1. Effect of enzyme hydrolyzates of three sources of protein on autolysis of *S. faecalis* 9790.

NOTE: The following concentrations were used: oatmeal, equivalent to 1.47 mg protein (or 21 μg *l*-lysine) per ml; whole egg, dried, equivalent to 0.42 mg protein (or 23 μg *l*-lysine) per ml; whole milk, dried, equivalent to 0.42 mg protein (or 32 μg *l*-lysine) per ml.

occurred rapidly after 6–8 hours. With egg hydrolyzate, moderate autolysis occurred, whereas with milk hydrolyzate, normal growth, without lysis, was maintained throughout the incubation period.

It was reported (7) that an excess of arginine, or threonine, or both in casein or egg albumin hydrolyzates may create an amino acid imbalance and thus inhibit the utilization of lysine by *S. faecalis*. It seemed possible, however, that autolysis could account for the apparent amino acid antagonisms. The effect of the addition of arginine or lysine to milk hydrolyzate, and of threonine

or lysine to egg hydrolyzate is shown in Fig. 2. Lysis did not occur with milk hydrolyzate alone, but growth was increased by the addition of lysine, indicating that the concentration of lysine in the milk hydrolyzate was high enough to prevent autolysis but was not sufficient to give maximum growth. When arginine was added, growth appeared to be increased, followed by gradual lysis. Although slight autolysis occurred with egg hydrolyzate alone, the addition of lysine increased growth and prevented lysis, whereas threonine magnified the degree of lysis.

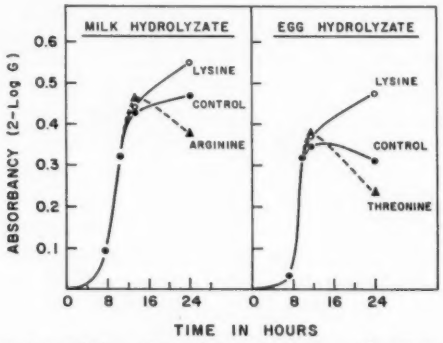

FIG. 2. Effect of the addition of *l*-arginine, *l*-lysine, and/or *dl*-threonine on autolysis of *S. faecalis* 9790 with enzyme hydrolyzates.

NOTE: The following concentrations were used: *l*-arginine or *l*-lysine, 5 μg per ml (or per 0.16 mg protein); *dl*-threonine, 7.5 μg per ml (or per 0.16 mg protein).

Experiments with L. mesenteroides

Enzyme Hydrolyzates

Foods, after enzyme hydrolysis, were evaluated by a titrimetric assay in the basal medium of Horn, Blum, and Womack (9) and in the same medium

TABLE I

Comparison of P.E.R. values and bacteriological values found with *L. mesenteroides* and enzyme hydrolyzates in simple or complex media

Source of protein	P.E.R.		Bacteriological value	
	Assay value	% of egg	Medium A*	Medium B†
Dried whole egg	3.35	100	100	100
Oatmeal + milk	2.90	87	87	120
Dried whole milk	2.56	76	71	120
Casein	2.50	75	61	110
"Protein cereal" F	2.21	66	102	—
"Protein cereal" B + milk	2.19	65	65	82
"Protein cereal" M	2.13	64	97	92
Oatmeal	2.13	64	88	92
Wheat germ cereal	1.59	48	52	52
"Protein cereal" B	0.03	0.9	49	43

*Medium of Horn, Blum, and Womack (9).

†Same as medium A, but supplemented with amino acids non-essential for the rat. The following concentrations were used per 100 ml double strength basal medium: *dl*-aspartic acid 12 mg, *dl*-glutamic acid 47 mg, *dl*-alanine 4 mg, glycine 20 mg, *l*-proline 7 mg, *l*-hydroxyproline 1 mg, *dl*-serine 12 mg, *l*-tyrosine 7 mg, *dl*-histidine 6 mg. Essential amino acids added to the medium included *l*-arginine.HCl 32 mg, *dl*-tryptophan 40 mg, per 100 ml 2× strength basal medium.

supplemented with amino acids not essential for the rat. Bacteriological values for 10 foods were compared with P.E.R. values for the same samples. The results are shown in Table I. In the basal medium of Horn *et al.* (Medium A) bacteriological values for several foods agreed with P.E.R. values while results for oatmeal or mixed cereals did not agree. Amino acid determinations (7) showed that arginine was the most limiting amino acid in hydrolyzates of dried whole egg, casein, oatmeal, "protein cereal" M, and "protein cereal" B.

Bacteriological values were also compared with P.E.R. values after supplementation of the basal medium with arginine and trytophan and amino acids not essential for the rat. Results obtained with the supplemented medium (Medium B) are shown in the last column in Table I. Under these conditions the bacteriological values correlated poorly with P.E.R. values. Formol titration of the hydrolyzates indicated that the proteins of the foods were incompletely digested by the proteolytic enzymes. The extent of digestion ranged from about 50% for dried whole egg to less than 30% for "protein cereal" M.

Acid Hydrolyzates

Horn and Blum reported that a 2-hour acid hydrolysis resulted in the maximum release of cystine from the proteins of foods. However, information on the effect of time of hydrolysis on the microbiological availability of other amino acids was given only for the proteins of whole wheat and coconut globulin. It was therefore of interest to determine the effect of hydrolysis time on the relative availability of amino acids e.g. lysine and methionine from the proteins of a variety of foods of plant and animal origin. Using hydrolyzed whole egg powder as reference standard, the microbiologically available lysine was determined in duplicate assays on each of five sources of cereal protein after 2 and 8 hours' hydrolysis. Methionine values for five sources of animal protein were also determined in a similar manner. As shown in Table II,

TABLE II

Effect of hydrolysis time on assays for lysine and methionine with *L. mesenteroides*

Source of protein	Lysine (% of egg)				Source of protein	Methionine (% of egg)			
	2 hour		8 hour			2 hour		8 hour	
	(1)	(2)	(1)	(2)		(1)	(2)	(1)	(2)
"Protein" bread	45	45	43	44	Cheddar cheese (dried)	99	92	103	99
Cracked wheat bread	37	38	37	38	Hamburger (dried)	87	86	84	87
Rolled flour rolls	18	18	19	18	Casein	113	107	109	106
Rolled oats	34	33	31	31	Fish flour	123	118	117	115
Whole wheat flour	57	58	54	54	Whole milk	93	87	86	85

NOTE: Acid hydrolyzates were used throughout. The whole egg standard was also hydrolyzed for 2 or 8 hours.

values for lysine or methionine after 8 hours did not differ significantly from values at 2 hours for any of the foods tested, and showed that the rate of release of lysine or methionine, relative to whole egg, was unaffected by the time of hydrolysis.

The effect of more complete release of amino acids on the bacteriological estimates of relative nutritive value was investigated using 2-hour acid hydrol-

yzates (13) of foods and the medium of Horn *et al.* (9) supplemented with amino acids in the amounts shown in Table I. Bacteriological values were determined together with estimates of the lysine and cystine concentration of each sample expressed as a percentage of the concentration in whole egg. The results were compared with P.E.R. values for the same samples as shown in Table III. The foods were placed in two groups: those with an apparent deficiency of either lysine or cystine. With foods low in lysine, a significant relationship was found between concentration of lysine and bacteriological value and between concentration of lysine and P.E.R. as shown in Fig. 3 by the regression lines A and B, respectively. With foods low in cystine, the cystine estimates correlated closely with bacteriological values but did not agree with P.E.R.'s as shown in Fig. 3 by the regression lines C and D, respectively.

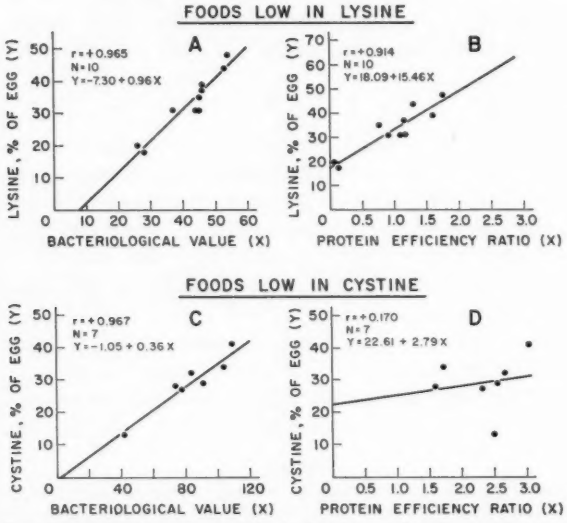

FIG. 3. Correlation between bacteriological value and amino acid concentration and between protein efficiency ratio and amino acid concentration for foods low in lysine (A and B); and for foods low in cystine (C and D).

Effect of Limiting Essential Amino Acids on Growth and Relative Protein Value

The results of the preceding experiments suggested that the growth response of the test organism to protein hydrolyzates may be directly proportional to the concentration of the most limiting essential amino acid. An attempt to confirm this hypothesis was made by measuring the effect of the addition of each of the four most limiting essential amino acids of whole egg powder, whole milk powder, or a mixed protein cereal, on the growth response of *L. mesenteroides* to acid hydrolyzates of each source of protein. The results are shown in Table IV. With each of the three protein sources, a significant increase in growth occurred only on the addition of the most limiting amino acid. Other amino acids had little or no effect on the growth response of *L. mesenteroides*.

TABLE III

A comparison of bacteriological values and P.E.R. values for foods low* in lysine or cystine

Source of protein	P.E.R. Assay value	P.E.R. % of egg	Bacteriological value (*L. mesenteroides*)	Lysine,† %	Cystine,† %
Dried whole egg (std.)	3.35	100†	100	100	100
Foods low in lysine					
Oatmeal + wheat gluten	1.75	52	54	48	145
Wheat germ cereal	1.59	47	46	39	76
"Protein" bread	1.29	39	53	44	72
Whole wheat flour	1.17	35	44	31	82
Roman meal bread	1.16	35	46	37	75
Whole wheat bread	1.09	33	45	31	61
Wheat gluten bread	0.90	27	37	31	77
White bread	0.77	23	45	35	80
Wheat gluten rolls	0.13	3.9	28	18	87
"Protein cereal" B	0.03	0.9	26	20	49
Foods low in cystine					
Fish flour	3.04	91	114	160	41
Dried hamburger	2.68	80	84	114	32
Dried whole milk	2.56	76	91	116	29
Casein	2.50	75	42	136	13
Dried cheddar cheese	2.32	69	78	133	27
Lima beans (cooked)	1.72	51	104	115	34
White wax beans (cooked)	1.59	47	74	102	28

NOTE: Acid hydrolyzates (2 hr) were used throughout.
*Relative to the concentration in whole egg.
†P.E.R. value for whole egg was arbitrarily set at 100.

TABLE IV

Effect of supplementation with most limiting essential amino acids on the response of *L. mesenteroides* P-60 to acid hydrolyzates of foods

Source of protein	Amino acid addition*: Lysine	Isoleucine	Threonine	Valine	Cystine	Growth response, %	Order of most limiting essential amino acids†
Dried whole egg	−	−	−	−	−	100	
	+	−	−	−	−	113	(1) Lysine
	−	+	−	−	−	101	(2) Isoleucine
	−	−	+	−	−	103	(3) Threonine
	−	−	−	+	−	100	(4) Valine
Dried whole milk	−	−	−	−	−	100	
	+	−	−	−	−	106	(3) Lysine
	−	+	−	−	−	97	(2) Isoleucine
	−	−	+	−	−	100	(4) Threonine
	−	−	−	−	+	115	(1) Cystine
Mixed protein cereal	−	−	−	−	−	100	
	+	−	−	−	−	115	(1) Lysine
	−	+	−	−	−	99	(3) Isoleucine
	−	−	+	−	−	102	(4) Threonine
	−	−	−	+	−	102	(2) Valine

*The following concentrations were used: *l*-lysine 50 μg, *l*-isoleucine 25 μg, *dl*-threonine 50 μg, *l*-valine 25 μg, *l*-cystine 12.5 μg/mg protein.
†Determined by method of Halevy and Grossowicz (7).

In amino acid assays by bacteriological methods, it is known that the shape of the response curve depends upon the particular amino acid being measured (19, 20). Since the growth response of the test organism in bacterial methods of protein evaluation is apparently determined by the most limiting amino acid in each hydrolyzate, a serious drift in assay values may be expected with foods in which the most limiting amino acid differs from that in the whole egg standard. It was, therefore, of interest to determine the effect of different limiting essential amino acids on the estimates of bacteriological value with *L. mesenteroides*. Acid hydrolyzates of whole milk powder, casein, and oatmeal were studied over a range of dosage levels in repeated assays with hydrolyzed whole egg powder as reference standard. As shown in Table V, a significant drift in values occurred with milk and casein, in which the limiting amino acid was cystine, whereas with oatmeal, limiting in lysine, the drift in values was negligible. Since lysine was the factor limiting growth of the test organism in the egg standard, it was concluded that the bacteriological assay procedures are valid only when the response to sample and standard is influenced by the same limiting amino acid.

TABLE V

Effect on differences in limiting essential amino acids on bacteriological values with *L. mesenteroides* at different dosage levels

Source of protein	Limiting essential amino acid	Assay	Bacteriological value, % of egg							
			Dosage level (mg protein/ml)							
			0.0	0.4	0.8*	1.2*	1.6*	2.0	2.4	2.8
Milk	Cystine	1	0	60	70	80	81	84	87	116
		2			81	104	114			
		3			94	98	100			
		4			87	93	102			
Casein	Cystine	1	0	35	45	50	52	50	52	56
		2			39	44	58			
		3			30	37	41			
		4			44	52	56			
Oatmeal	Lysine	1	0	62	61	59	60	60	63	65
		2			69	57	67			
		3			68	66	72			
		4			71	70	75			

*Working dosage level.

Discussion

The autolytic effect observed with *S. faecalis* 9790 is in agreement with the findings of Toennies and Gallant (17) and of Toennies and Shockman (18), who used synthetic media. The addition of arginine to milk hydrolyzate and of threonine to egg hydrolyzate caused a slight increase in growth of *S. faecalis*, but was followed by gradual lysis. This initial increase in cell population evidently created an intracellular dilution of the lysine concentration with concurrent lysis of the cells. From these observations it was concluded that *S. faecalis* was unsatisfactory for the nutritional evaluation of food proteins or, as suggested by Halevy and Grossowicz (7), for the study of imbalances among amino acids.

The evaluation of enzyme hydrolyzates of protein in foods with *L. mesenteroides* P-60 gave results which did not agree consistently with P.E.R. A sequence of gastric and intestinal proteinases, designed to simulate human digestion, gave inadequate hydrolysis of the food proteins. Many foods, particularly cereals, contain a high proportion of carbohydrate and other nonprotein material which, by enveloping the protein, may have prevented the enzyme from obtaining adequate contact with the protein fractions of the samples. The possibility cannot be excluded that autodigestion of the proteolytic enzymes may have contributed amino acids to the hydrolyzates in concentrations which could distort the estimates of protein quality (21).

With arginine omitted from the medium, the results suggested that the *L. mesenteroides* method (with enzyme or acid hydrolyzates) was, essentially, an arginine assay. In support of this view, *L. mesenteroides* is known to have a high requirement for arginine (12). Furthermore, in the present study. cereal proteins, which contain substantial amounts of this amino acid, gave high values in comparison with P.E.R.'s when assayed in the medium of Horn, Blum, and Womack (9) from which arginine was omitted. When its requirement for arginine was satisfied, *L. mesenteroides* showed a relatively high requirement for lysine with a lower requirement for cystine and methionine. Cystine has an apparent sparing effect upon the methionine requirement of the rat although it is not essential for growth. Furthermore, the methionine–cystine relationship observed with rats does not occur with *L. mesenteroides*. This inherent difference in amino acid requirements was evidently largely responsible for the lack of agreement between the bacteriological method and P.E.R. for legumes and foods of animal origin.

The relationship between lysine concentration and P.E.R. shown with cereals suggested, in agreement with the work of others (22, 23), that the quality of proteins deficient in lysine may be predicted directly from their lysine content.

Although it was shown by formol titration that 80–90% of the amino acids were released from the proteins of foods after an 8-hour acid hydrolysis, the bacteriological values obtained were similar to those determined with 2-hour acid hydrolyzates in which 60–70% of the amino acids had been released. These observations are in agreement with the work of Horn and Blum (13), who reported that, after a 2-hour acid hydrolysis, all of the amino acids of coconut globulin or whole wheat proteins were either free in solution or were in a peptide form small enough to be assayed with *L. mesenteroides* P-60.

Evidence was obtained to show that the growth response in the bacteriological methods was influenced only by the most limiting amino acid relative to the growth requirements of the test organism. Supplementation with single amino acids other than the one most limiting did not affect the response of *L. mesenteroides* to hydrolyzates of foods. In addition, the use of bacteriological methods for protein evaluation appeared to be invalidated by the drift in values observed in assays with *L. mesenteroides* and hydrolyzates of proteins deficient in an essential amino acid other than the one limiting for growth of the test organism in the whole egg standard.

Although the methods employed in this study may be useful in the detection of changes in the nutritive value of a food resulting from heat processing or other physical or chemical treatment, it was concluded that bacteriological assay techniques are unsatisfactory as screening procedures for predicting the quality of protein in a wide range of foods.

The relationship observed between amino acid concentrations and P.E.R. suggested that a satisfactory method of protein evaluation, based on the chemical score of Block and Mitchell (24), might be achieved by the determination of fewer amino acids. This approach is discussed in a subsequent report by McLaughlan *et al.* (25).

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THE ACETYLATION OF THROMBIN[1]

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Abstract

Purified thrombin-C loses its clotting power upon acetylation. The thrombin-E which is produced during the acetylation has approximately twice the proteolytic activity as the original thrombin-C. Evidently amino groups are not necessary to have thrombin-E activity, but if *o*-acyl groups are also produced the enzyme does not hydrolyze *p*-toluenesulphonylarginine methyl ester (TAMe). The activity can be recovered by spontaneous hydrolysis of the *o*-acyl groups at pH 8.5. Thrombin-E does not activate fibrinogen, but does lyse fibrin. The optimum pH with TAMe as substrate is 8.8. It may be that thrombin-C is a dimer of the basic structure in thrombin-E.

Introduction

In the activation of prothrombin there is an orderly sequence of events consisting of degradation of the molecule (1). Toward the end of the interactions thrombin activity appears. This is generally recognized by the clotting of blood, plasma, or purified fibrinogen. Just before this the proteolytic potential of prothrombin is measurable. After thrombin activity has developed it may disappear while proteolytic activity remains (2, 3). There are two measurable functions of thrombin. One is the activation of fibrinogen (thrombin-C) and the other the proteolysis of fibrin. The latter is also measured as hydrolysis of *p*-toluenesulphonylarginine methyl ester (thrombin-E). It seems as though thrombin-C arises from thrombin-E and that this is a reversible process

$$\text{thrombin-E} \rightleftarrows \text{thrombin-C}.$$

There are two distinct enzyme functions, and two corresponding structures are needed to account for these activities. Perhaps one of the enzymes differs from the other simply by a few atoms or only in spatial configurations. Another alternative would be that a fundamental enzyme unit of prothrombin would pair with a similar one also derived from prothrombin. Prothrombin is large enough to give rise to two thrombin-E molecules from which one thrombin-C molecule could form. This infers duplication of structure in prothrombin. As a step in the direction toward getting experimental information to evaluate these ideas we felt the need to get thrombin-E in purified form and in suitable quantities for the study of its properties.

In our work we discovered that acetylation of thrombin-C destroys its clotting power. Simultaneously the esterase activity not only remains but approximately doubles in strength. Among other things the acetylation covers the amino groups and if carried too far *o*-acyl groups form and the esterase activity is also lost. This is, however, reversible for we can recover the esterase activity. The acetylated thrombin does not activate purified fibrinogen, but does use fibrin monomer and fibrin as a substrate.

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Materials and Methods

The materials and methods used in this work have been described previously: (*a*) purified resin thrombin (4); (*b*) TAMe assay and fibrinogen assay for thrombin (5, 6); purification of fibrinogen (7); ninhydrin reaction for amino groups (8); hydroxylamine reaction for the determination of *o*-acyl groups in proteins (9, 10); and color reaction for tyrosine (11). To compare TAMe units of thrombin with clotting units the reagents for each of the two determinations were set for equivalent calibration when a provisional standard of thrombin was analyzed.

Experimental

Acetylation of Thrombin

In principle, the thrombin was dissolved in sodium acetate buffer and acetylated with the use of acetic anhydride. The procedure is described by a specific example. About 100,000 units (ca. 20 mg) of thrombin were dissolved in 10 ml of water. A saturated solution of sodium acetate was prepared at room temperature. The thrombin solution was put in an ice bath and while it was being stirred an equal volume of the sodium acetate solution was added slowly so as to keep the temperature at zero throughout the procedure. Glass electrodes were in the solution to help with the control of pH. Then acetic anhydride was added in portions from a small burette. A drop in pH from around 8.5 to 5.5 was observed with addition of about 0.36 ml of acetic anhydride in about half an hour. The time to stop was determined by pH, but was usually predictable from the quantity of acetic anhydride used. Below pH 5.5 cloudiness is observed and irreversible inactivation occurs.

When the acetic anhydride had been added the glass electrodes were removed but the stirring was continued and powdered ammonium sulphate (10.2 g) was added to bring the mixture to half saturation. This precipitated the thrombin-E, and it was centrifuged at 0° C. The precipitate was dissolved in 10 ml distilled water, and mixed with an equal volume of cold (−70° C) acetone. The precipitate was centrifuged down, dissolved in 10 ml water, and again precipitated with cold acetone. The precipitate was then again dissolved in 10 ml of cold water.

Events during Acetylation

During the acetylation it was easily possible to take samples and dilute them extensively so that the electrolytes would not interfere with assay for activity. Very early in the acetylation most of the thrombin-C activity was gone. The thrombin-E activity declined gradually as the pH dropped. At pH 5.5 about half of the thrombin-E activity remained. This was, however, only one way of observing the changes.

When aliquots were taken from the reaction mixture at different times and precipitated with ammonium sulphate and twice with acetone we found a loss of thrombin-C activity, but there was an increase in thrombin-E activity (Table I). The ammonium sulphate and acetone precipitations tend to hydrolyze the less stable acetylated groups. In successful thrombin acetylation the thrombin-E activity usually doubles and the thrombin-C activity dis-

appears to a point where only traces remain. This doubling of activity is evidently a number which exactly corresponds to a certain quantitative unfolding of structure.

We studied the acetylation rate by use of the ninhydrin reaction. This was applied to materials purified by ammonium sulphate and acetone precipitation. About half the amino groups were covered when the procedure was completed (Table I).

TABLE I

Acetylation of thrombin in sodium acetate

Acetylation, pH	Fibrinogen assay, units/ml	TAMe assay, units/ml	Acetylation, %
8.5	7,200	7,600	None
7.0	284	12,600*	42.3
6.3	±2	12,400*	47.5
5.6	None	10,800*	51.2

*Assay at pH 8.4. This is not optimum for acetylated thrombin.

The acetylation was also done with the use of a 15% (w/v) solution of potassium carbonate as buffer salt in place of sodium acetate. Under these conditions the acetylation was more extensive. We again purified by precipitation with ammonium sulphate and acetone. In one instance the acetylation was 96% as measured by the ninhydrin reaction (Table II). There was con-

TABLE II

Acetylation of thrombin in 15% potassium carbonate solution

			Acetylation	
Acetylation, pH	Fibrinogen assay, units/ml	TAMe assay, units/ml	% amino groups	*o*-acyl, micromoles
10.5	5625	5990	None	None
10	3.6	9600*	—	None
9.5	None	7200*	17	None
9.1	None	6100*	66	0.07
8.3	None	6100*	87	0.16
7	None	6000*	95	0.16
6.5	None	600*	96	0.20
The thrombin was precipitated with ammonium sulphate and acetone, taken up in water, and the pH was changed to 8.5 by adding alkali. Then the recovery of thrombin-E activity was followed. The time in minutes is given by figures in column to left. All other figures correspond to the respective column headings.				
30	None	600*	—	0.18
60	None	1200*	—	0.03
90	—	—	—	+(<0.02)
180	None	2100*	—	None
240	None	3000*	—	None

*Assay at pH 8.4. This is not optimum for acetylated thrombin.

siderable esterification of hydroxyl groups giving *o*-acyl structures detected by the hydroxylamine reaction. This, however, was associated with a loss of thrombin-E activity. In a water medium at pH 8.5 this activity returned in appreciable strength (Table II) and at the same time the *o*-acyl groups hydro-

lyzed. Most likely serine, hydroxyproline, and threonine are involved in these reactions (10).

Some Properties of Acetylated Thrombin

One of the most conspicuous properties of acetylated thrombin is its failure to activate fibrinogen. No clot forms, but the fibrinogen retains its property of being activated by thrombin-C. On the other hand thrombin-E is proteolytic with fibrin as substrate. In this respect it is similar to thrombin-C (Fig. 1).

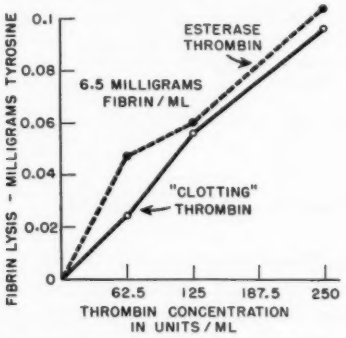

FIG. 1. The amount of fibrin lysed in the presence of various amounts of thrombin-C or thrombin-E at pH 8.5 and 28° C.

The experiments supporting these assertions consisted of the following: fibrin was produced by clotting a purified fibrinogen solution. A minimum amount of thrombin was used for the clotting. The clot was thoroughly washed, dried at 60° C for 24 hours, and powdered. The powder was suspended in buffer (2-amino-2(hydroxymethyl)-1,3-propanediol) at pH 8.5. This was filtered through gauze to remove gross particles. Then thrombin was added and the mixture was shaken 4 hours at 28° C. The solution was then centrifuged at 106,000 *g* and the tyrosine concentration in the supernatant solution was measured. A correction was made for the color contributed by thrombin.

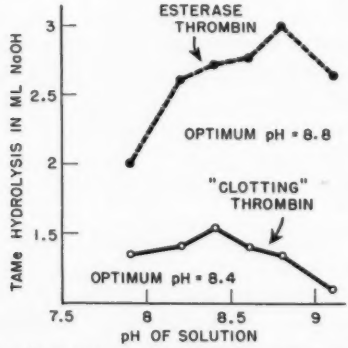

FIG. 2. The hydrolysis of TAMe in Tris buffer at 37° C. The protein concentration for the two enzymes was the same.

In the thrombin-E solution the soluble tyrosine was proportional to enzyme concentration and approximately equal to that found in the thrombin-C solutions. The reaction is apparently first order with respect to thrombin.

The optimum pH for thrombin-E activity is near pH 8.8 as compared with thrombin-C which is near pH 8.4 (Fig. 2). In the actual experiments the thrombin-E was derived from the same thrombin-C that was used in the assay and diluted to the same original protein concentration. In this way the optimum activity for each enzyme can be used as a point of comparison. The thrombin-E activity is double that of the thrombin-C when the protein concentration is the same as under the conditions of our experiment.

Discussion

Ever since Ware and Guest (12) observed in this laboratory that thrombin lyses fibrin there have been questions raised about the accuracy of the view. It is an important point to be considered because of the implications about the nature of thrombin and the way it functions in the clotting of fibrinogen, in the viscous metamorphosis of platelets, in the activation of prothrombin, in the activation of Ac-globulin, etc. The common question about the purity of the fibrin or of the thrombin has been raised repeatedly. We think this objection is wearing quite thin now. In our work the original prothrombin was "pure" by several criteria and it did not lyse the fibrin. The thrombin produced from the prothrombin did lyse the fibrin and the thrombin-E which was a further derivative lysed it even more effectively. Another enzyme would have to follow prothrombin in a series of fractionations that takes 2 days, be activated by those substances that activate prothrombin, course through another IRC-50 column with thrombin, be resistant to acetylation, and double its activity upon acetylation.

The synthetic production of thrombin-E opens a new field in blood coagulation research. Among other opportunities its pharmacology can be studied. Since esterase activity is the first to appear in prothrombin activation it may be that this activity is even of more interest than the commonly observed clotting function of thrombin. In this direction of study we may be able to uncover unrecognized functions of prothrombin in metabolism.

The methods we used for acetylation are known to be specific for amino groups (10). These are needed to have thrombin-C (13), but apparently not for thrombin-E. When, however, the hydroxyl groups were esterified, in the potassium carbonate medium we used, there was loss of activity due to reversible alterations. This probably is concerned collectively or separately with serine, hydroxyproline, or threonine. When diisopropylphosphorofluoridate is used to inactivate thrombin it attaches to serine (14). The phenol group on tyrosine is excluded because the color reaction for tyrosine did not change upon acetylation.

By blocking the amino groups and getting thrombin-E the acidic groups are favored in their ionization and the optimum pH shifts to the basic side in going from thrombin-C to thrombin-E. Uncovered amino groups probably depress the activity of the acidic groups in the functioning of the enzyme, but we also

need to account for the increase in esterase activity. It could be that thrombin-C is a dimer and upon further degradation there is a simpler monomer that has only proteolytic activity. A preliminary determination of sedimentation constant in the ultracentrifuge gave data to fit this idea. Moreover, the loss of thrombin-C activity occurs long before very many amino groups are acetylated. A very sensitive bond in thrombin-C is opened at the very beginning. The group(s) occupied in holding the dimer together can become free and give rise to twice the previous esterase activity. The doubling of activity is not easily accounted for on another basis.

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STUDIES ON AROMATIC ESTERASE AND CHOLINESTERASE OF HUMAN SERUM[1]

A. MARTON AND W. KALOW

Abstract

Human serum proteins were separated by electrophoresis on filter paper. Cholinesterase could not be readily eluted from the paper but was demonstrated to be between α_2- and β-globulins by a staining method. Aromatic esterase migrated in close relation to albumin and could be eluted by $M/15$ phosphate buffer of pH 7.4. The esterase activity was demonstrated in the eluates by ultraviolet spectrophotometry, using phenylacetate as a substrate. Versene caused a strong inhibition of aromatic esterase activity in vitro. The activity could be restored by different cations of which Ca^{++}, Cu^{++}, and Mn^{++} were most potent. In 74 persons who were considered as normal controls in regard to the esterases, there was no correlation between cholinesterase and aromatic esterase activity. However, in 25 individuals suffering from cancer and/or liver dysfunction, a significant correlation between the two enzyme activities appeared.

Introduction

Those enzymes, which were formerly called common esterases, have been classified so that some attention must be paid to nomenclature. In 1942, Richter and Croft (1) designated a common esterase as ali-esterase. In 1949, it was first recognized that ali-esterases consisted of two distinct types of enzymes (2, 3); in 1953, Aldridge (4) characterized these enzymes in some detail and called them A and B esterase. Hence, in the literature prior to 1953, and occasionally in more recent publications, ali-esterase may mean either A or B esterase. However, as suggested by Mounter and Whittaker (5), the term ali-esterase is presently often used in a more restricted sense and means B esterase, while A esterase is called aromatic esterase. In contrast to many animal sera, human serum lacks the B or ali-esterase (5, 6, 7) but contains the A or aromatic esterase in addition to pseudocholinesterase (7, 8). The purpose of this paper is to present some comparisons and distinctions between these two esterases of human serum.

Experimental

Electrophoresis

The separation of serum proteins was carried out by electrophoresis using the procedure of Poulik and Smithies (9). Serum (20–80 microliters) was applied to paper strips (Whatman No. 1, 12–13 in. × 1 and ½ in.) soaked in 0.048 *M* barbiturate buffer of pH 8.55 (9). The same buffer filled the electrode compartments. A potential of 140–150 volts d-c. was applied for a period of 15–16 hours at room temperature. The serum proteins were stained with amido black 10 B (Bayer Leverkusen, Germany).

Measurements of Esterase Activity

1. Aromatic Esterase

The activity of aromatic esterase in the solution was measured by following the change of absorbance which occurred during hydrolysis of phenylacetate

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Contribution from the Department of Pharmacology, University of Toronto, Toronto, Ontario.

(10, 11). The Beckman recording spectrophotometer Model DK 2 was used. The reaction was conducted in absorption cells with a light path of 1 cm at a temperature of 24–26° C. During any series of measurements, the temperature in the absorption cells changed not more than 0.2° C, as determined with a thermistor. The duration of a single measurement varied between 3 and 30 minutes depending on the esterase activity.

Hydrogen ion concentrations were determined with Beckman pH meter Model G. Phosphate buffer (M/15, pH 7.4) was used as solvent, diluent, eluting agent, and optical blank in all the reported studies.

The sources of enzyme were samples of human serum which appeared to be free of haemolyzed erythrocytes. Fresh sera with only the normal type of cholinesterase (12) were used for this investigation. The final dilution of the crude serum used was 1:200. If serum had been subjected to electrophoresis on paper, the paper was dried, cut into suitable sections, and the aromatic esterase eluted by placing the pieces of filter paper into a buffer solution for 1 hour at room temperature. The amount of buffer solution was usually 3 ml, of which 2 ml was used as the eluate for determining esterase activity. For inhibition studies, 5 ml of buffer was used for eluting sections of paper.

The substrate was a freshly prepared solution of phenylacetate. Its initial concentration in the cell was 3.95×10^{-4} M. Tetraethylpyrophosphate (TEPP) and physostigmine (eserine) sulphate were dissolved in buffer shortly before use.

2. Cholinesterase Activity

Cholinesterase activity of diluted serum was measured by ultraviolet spectrophotometry using the procedure of Kalow and Lindsay (13). The enzyme on filter paper was located by a staining method described below.

Ravin *et al.* (14) have developed a staining method for the histochemical detection of cholinesterase. This method proved to be useful for locating the enzyme after electrophoresis on filter paper. The substrate was beta-carbonaphthoxy choline iodide. The dried paper was suspended for 1 minute in a solution containing 20 mg of substrate per 50 ml. Subsequently, a purple color was developed with the aid of naphthanil diazo blue B at the site of cholinesterase activity.

Results

1. Separation of A Esterase and Cholinesterase by Paper Electrophoresis

Figure 1 shows an example of the results obtained by a combination of the methods described above. Paper strips containing serum were stained with amido black after electrophoresis, and showed five dark bands which were designated (9) as albumin, α_1-, α_2-, β-, and γ-globulins. The cholinesterase could be detected on paper strips unstained by amido black. By dividing the paper strips and staining one part for total protein and the other part for cholinesterase, this enzyme was found to be located between the α_2-, and β-globulins. Several attempts to find a specific staining method suitable for localizing aromatic esterase activity on paper were unsuccessful. Esters of phenol and some derivatives of phenol are rapidly hydrolyzed by aromatic esterases but most methods of determining phenol require an alkaline medium.

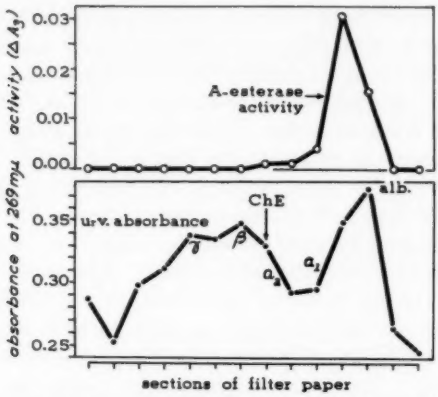

FIG. 1. Ultraviolet absorption and aromatic esterase activity of eluates of filter paper after electrophoretic separation of human serum proteins.

Abscissa: Sections of filter paper. The sections were equal and each was about 1 cm wide.

Lower curve: Absorbance at 269 mμ of the eluates prior to the addition of phenylacetate. The results of staining methods on paper were correlated with the measurements of absorbance; the locations of cholinesterase (ChE), of albumin (alb.), and of α_1-, α_2-, β-, and γ-globulins are indicated along the curve. The absorbance scale of the ordinate does not start at zero because the eluates absorbed some light in the absence of proteins; this absorption can be accounted for by the presence of barbital from the buffer used during the electrophoresis.

Upper curve: Aromatic esterase activity of the eluates. The ordinate scale indicates the rise of absorbance at 269 mμ during the first 3 minutes after the addition of phenylacetate to the eluates.

Alkalinity was found to cause spontaneous hydrolysis of phenol esters so that the enzymatic hydrolysis was masked; furthermore, sensitive staining methods for phenol always stained, to some extent, the proteins on filter paper. Thus the attempt was made to elute the aromatic esterase from filter paper, and to determine the enzymatic activity of the eluate in the spectrophotometer. This method was found to be suitable for the detection of aromatic esterase activity but cholinesterase activity could not be localized in this way, i.e. no eluate of any paper section hydrolyzed benzoylcholine.

The absorbance of the eluate was always measured with buffer as the optical blank prior to the addition of phenylacetate. The absorbances were highest in the eluate of paper sections containing albumin and β- and γ-globulins; no peaks of absorbance were noted in the range of the α-globulins.

Aromatic esterase migrated at a faster rate than cholinesterase, and could be eluted from paper. It did not hydrolyze benzoylcholine and beta-carbonaphthoxy choline. Most of the aromatic esterase activity was found in the albumin portion; however, the greatest density of albumin, as judged by its ultraviolet absorbance, and the peak of aromatic esterase activity did not coincide. This was confirmed by several elutions in which particularly narrow paper sections in the albumin range were examined.

When rates of hydrolysis of phenylacetate by 25 diluted crude sera and by the corresponding eluates were compared, the correlation coefficient was 0.94 indicating reproducibility of the method. However, the total amount of aro-

matic esterase recovered from sections of filter paper containing albumin was on the average only $11.4 \pm 0.9\%$ of the amount which appeared to be present in crude serum. There was no evidence for an incomplete elution of aromatic esterase from paper; at least the paper did not retain any ability to hydrolyze phenylacetate after routine elution. This was tested by placing eluted pieces of filter paper without the eluate at the bottom of an absorption cell, filling the cell with a solution of phenylacetate, and measuring the absorbance for prolonged periods of time. Since the buffer applied to the paper during the electrophoresis contained barbital, it was considered possible that the eluted esterase was inhibited by the presence of barbital. The concentration of barbital in the eluates could be estimated by its ultraviolet absorbance; the concentration varied with the size of the eluted piece of paper but was usually in the order of 10^{-3} *M*. Barbital in concentration of 8×10^{-3} *M* caused 50% inhibition of aromatic esterase. This could not explain the apparent poor recovery of aromatic esterase. Hence it was tested whether an activator of aromatic esterase had been removed during the electrophoresis. It was found that the addition of Ca^{++} to the eluate enhanced the enzyme activity. Therefore, the following tests were performed.

2. Activators of Aromatic Esterase

The chelating agent ethylene diamine tetraacetic acid (EDTA, Versene) in concentrations of 5×10^{-5} *M* and 1×10^{-4} *M* caused complete inhibition of aromatic esterase. When the concentration of Versene was 2.5×10^{-5} *M*, only 6% of the original activity was blocked. Versene in these concentrations had no effect on the hydrolysis of benzoylcholine.

Different cations were capable of reactivating the A esterase activity completely blocked by Versene (1×10^{-4} *M*). Table I shows the results obtained by metal ions.

TABLE I

Reactivation of aromatic esterase previously blocked by 0.1 m*M* Versene

Cation	Relative* rate of hydrolysis of phenylacetate: With 2.5×10^{-4} *M* ion concentration	With 2.5×10^{-6} *M* ion concentration
Ca^{++}	164	144
Zu^{++}	35	0
Mg^{++}	11	0
Cu^{++}	88	0
Mn^{++}	76	3

*Original activity 100%.

3. Comparison of Aromatic and Cholinesterase Activities in Individual Sera

Aromatic and cholinesterase activities were determined without electrophoresis in sera of two groups of patients. One group consisted of 74 patients of a mental hospital. All these patients were adults with various mental disturbances; on the basis of previous studies (10), they were considered as normal controls in regard to esterases. The second group consisted of 25 patients suffering from cancer and/or liver disease. The results of the individual

determinations are shown in Fig. 2; the statistical parameters are indicated in Table II.

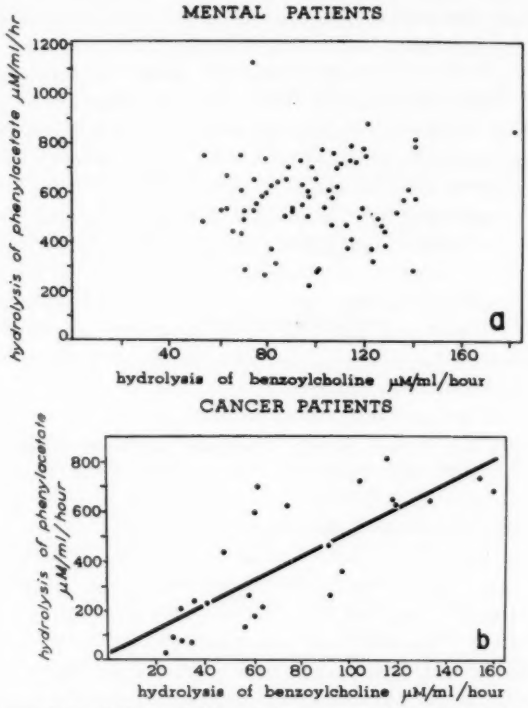

FIG. 2. Comparison of the activities of A esterase and cholinesterase in the sera of two groups of patients. The activity of A esterase is indicated by the rates of hydrolysis of phenylacetate, that of cholinesterase by the rates of hydrolysis of benzoylcholine. All reaction rates are expressed as micromoles of substrate hydrolyzed by 1 ml of serum per hour (cf. Table I).

(*a*) Mental patients: There is no correlation between the two enzyme activities. The same lack of correlation has been observed in healthy persons.

(*b*) Cancer patients: Both average enzyme activities are decreased and a significant correlation between the two activities can be noticed. The straight line was calculated by the method of least squares.

TABLE II

Person-to-person variation of A esterase (= aromatic esterase) and cholinesterase activities of serum

		Mental patients	Cancer patients
Cholinesterase activity* = x	Mean ± S.E.	99.9 ± 2.9	75.6 ± 8.0
	Coefficient of variation†	24.6%	52.9%
A esterase activity* = y	Mean ± S.E.	571.0 ± 18.2	387.2 ± 50.8
	Coefficient of variation†	27.5%	65.3%
Ordinate intercept = a		+542	+15.2
Slope = b		+0.29	+4.92
Correlation coefficient = r_{xy}		+0.04	+0.77

NOTE: Statistical parameters of data shown in Fig. 2 (linear regression $y = a + bx$). The data on mental patients are meant to represent normal control values.

*Micromoles of substrate hydrolyzed by 1 ml of serum per hour.

†Standard deviation expressed as percentage of mean.

Discussion

Enzymes (excluding aromatic esterase) that can hydrolyze phenylacetate are: ali-esterase (B esterase according to the nomenclature of Aldridge) and acetylcholinesterase. Ali-esterase is insensitive to inhibition by eserine but susceptible to alkyl phosphate inhibitors (4, 5, 7, 15), while cholinesterase is readily blocked by both types of inhibitors (16). Acetylcholinesterase, as well as ali-esterase, occurs in erythrocytes but the investigated samples in the present work were free of haemolyzed cells. Furthermore, the enzyme which hydrolyzed phenylacetate could not be blocked at all by TEPP and therefore was neither ali-esterase nor cholinesterase. Resistance to inhibition by eserine and alkyl phosphates is characteristic of aromatic esterase (4, 5). It should be emphasized that this observation confirms previous reports (5, 6, 7) that ali-esterase is not a normal constituent of human serum. Mounter and Whittaker (5) and Augustinsson (7) found a slight degree of hydrolysis of phenylacetate by pseudocholinesterase of human serum; this has escaped detection under the experimental conditions reported here.

Augustinsson (7) used electrophoresis on a cellulose column to determine the esterase-containing fractions of serum. He found human cholinesterase between the α_2- and β-globulins, and aromatic esterase close to the albumin fraction. Our results are in agreement with his. Cholinesterase of horse serum was first separated with the aid of paper electrophoresis by Gregoire and Derrien (17), who described the enzyme as being part of the α_2- and β-globulins. Tongi and Meier (18) reported that human serum cholinesterase migrated with the β-globulins. Pinter (19) applied beta-naphthylacetate to paper after electrophoresis and observed hydrolysis of this substrate in two areas; one of these areas was close to the α_2-globulins, the second area was in the range of albumin. Hence, the location of enzymes observed here is the same as that reported by Pinter, and one can conclude that beta-napthylacetate is hydrolyzed by both aromatic esterase and cholinesterase. Pinter designated the enzymes in the albumin fraction as ali-esterase but he did not employ means for distinguishing between ali- and aromatic esterase.

The aromatic esterase activity obtained by eluting filter paper was only a little more than 10% of the activity of serum. This could be explained by a loss of calcium and perhaps other ions during electrophoresis. The phosphoryl phosphatase of human serum, which is capable of hydrolyzing, for example, diisopropyl fluorophosphate, is another esterase of human serum which requires metal ions as activators; however, in contrast to aromatic esterase, this phosphatase is not activated by Mn^{++} (20, 21).

Lindsay (10) previously investigated in this laboratory the hydrolysis of various esters in the sera of numerous healthy subjects. There was a close correlation between the hydrolysis rates of various cholinesters and local anaesthetics but there was no correlation with the hydrolysis rate of phenylacetate. The esterase activities of the mental patients described in the present paper were very similar to those of the healthy persons previously investigated (10); particularly, there was the same lack of correlation between the hydrolysis rates of benzoylcholine and phenylacetate in individual sera. The significant

correlation in patients suffering from cancer and/or liver disease was apparently caused by a reduction of both esterase levels in the sera of some individuals. Cachexia and dysfunction of the liver have long been known to cause a lowering of cholinesterase levels (22). Hence it appears that these same factors cause a fall of aromatic esterase levels. A correlation between levels of cholinesterase and levels of albumin also seems to exist in patients with liver disease (23). Thus, the lowering of esterase levels under pathological conditions might be a non-specific sign of a reduced rate of protein synthesis in the liver. This conclusion is not necessarily invalidated by the reported increase of α_2-globulins in cancer patients (24). The protein responsible for most of this increase seems to be haptoglobin and it has been suggested that the levels of this protein are elevated by the breakdown of tissue (25).

Acknowledgment

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DIET AND CHOLESTEREMIA

III. EFFECT OF DIETARY PROTEINS WITH PARTICULAR REFERENCE TO THE LIPIDS IN WHEAT GLUTEN[1]

NARINDAR NATH, A. E. HARPER, AND C. A. ELVEHJEM

Abstract

The effects of different dietary levels of several proteins on the serum cholesterol concentration of the rat have been determined. With diets containing casein, serum cholesterol concentration was lowest when the protein level was 30–40%. The cholesteremic effects of fibrin and pork were similar to that of casein. Zein exerted a marked hypercholesteremic effect which could be counteracted by replacing it in part with casein. A soybean protein (Drackett) fed at high levels led to lower, and fed at lower levels, led to higher serum cholesterol concentrations than were observed with comparable levels of casein.

Wheat gluten fed to rats on a hypercholesteremic regimen caused a marked lowering of serum cholesterol concentration. However, when cholesterol and cholic acid were excluded from the diet, the substitution of wheat gluten for casein as the dietary protein caused a rise in serum cholesterol concentration. Extraction of wheat gluten with absolute ethanol led to the separation of lipid-like material possessing cholesterol-lowering activity. The extracted wheat gluten had a marked hypercholesteremic effect.

A high intake of the proteins of mechanically defatted beef or casein and skimmed milk was early claimed to induce atherosclerosis in the rabbit (1, 2). However, Freyberg (3), after observing that rabbits fed a diet containing a high level of vegetable proteins for as long as 11 months failed to develop atherosclerosis or hypercholesteremia, suggested that the effect observed previously (1, 2) might be due to some non-protein constituent of the diet. Later Meeker and Kesten (4) reported that a diet high in casein induced hypercholesteremia and atherosclerosis in rabbits whereas a high-protein diet containing primarily soybean flour had the opposite effect. In contrast soybean proteins, low in sulphur amino acids, have been shown to induce atherosclerosis and hypercholesteremia under other experimental conditions in the monkey (5) and the rat (6). A high level of dietary protein has been reported to cause a rise in the serum cholesterol concentration of the hereditarily obese mouse (7) but to reduce the accumulation of serum cholesterol in the normal mouse (7) and also in the rat fed a hypercholesteremic diet (8, 9). A high intake of protein is stated to be without effect on the serum cholesterol concentration of man (10) and similar results have been obtained in rabbits maintained on rations restricted in calories (11).

The ingestion of a diet containing 25% of hydrogenated coconut oil, 1% of cholesterol, and 0.5% of cholic acid leads to a rapid and marked elevation of serum cholesterol concentration in the rat (12). This regimen is suitable for the study of effects of dietary constituents on serum cholesterol concentration,

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and is currently being used in this laboratory in a study of the effects of different levels of some proteins of both animal and vegetable origin on the concentration of cholesterol in the serum of the rat. A preliminary report was published earlier (13).

Experimental

Male weanling rats of the Sprague–Dawley, Holtzmann, or Rolfsmeyer strains weighing 40–55 g were used in these experiments. There were six rats in each group.

The basal diet contained hydrogenated coconut oil, 25%; salts, 4%; cholesterol, 1%; cholic acid, 0.5%; and adequate quantities of all known vitamins (12). Sucrose was the carbohydrate and all changes in the diet were made at the expense of sucrose. The values for the nitrogen content of each of the proteins studied, as determined by the Kjeldahl method, were as follows: casein, 14.0%; pork, 15.1%; soybean protein,[2] 13.4%; zein, 14.5%; and wheat gluten 13.6%. The experiments were limited to 3 weeks' duration because a marked elevation of serum cholesterol concentration occurred within this period. Blood was drawn from individual rats under ether anesthesia by cardiac puncture after a 15- to 18-hour fasting period. The rats were then killed, their livers removed, weighed, and immediately frozen. In these experiments the serum cholesterol concentration of each rat was determined by the method of Henly (14). Total food consumption for each group was measured and in some experiments feces were collected and pooled samples analyzed for total lipids and Liebermann-Burchard-positive sterols.

Further details of the methods and analytical techniques have been described (12).

Results and Discussion

The effects of different dietary levels of casein on serum cholesterol concentration are shown in Fig. 1. Each increase in the casein level from 6% to 40% of the diet caused a progressive lowering of serum cholesterol concentration, but a further increase in the casein level to 69.5% of the diet caused the serum cholesterol concentration to rise. There was an inverse relationship between average daily food intake or average weight gain and serum cholesterol concentration (Fig. 1). Changes in serum lipid phosphorus concentration almost paralleled the changes in serum cholesterol concentration. No consistent relationship between the dietary level of casein and the concentrations of liver constituents was observed, but the concentrations of both total lipids and total cholesterol in the livers of rats fed the diet containing 40% of casein were slightly lower than those of the other groups.

Since diets with a protein content higher than 40% are rare, except experimentally, some other proteins were tested and compared with casein at the 40% and 10% levels. The results are shown in Table I. On comparing the cholesterol values in Table I with those in Fig. 1, it is seen that the serum cholesterol concentrations of rats consuming casein were higher in this experi-

[2]Drackett Assay Protein, The Drackett Product Company, Cincinnati, Ohio.

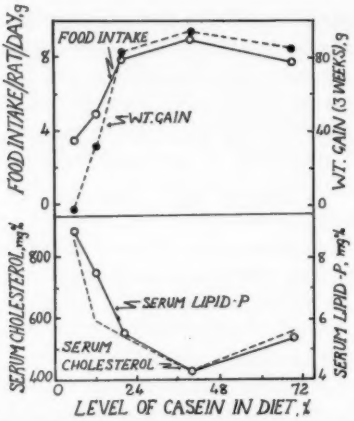

FIG. 1. Effect of different levels of casein in the diet on food intake, weight gain, and serum cholesterol and lipid phosphorus concentrations in the rat.

TABLE I

Effects of different dietary proteins on serum cholesterol concentration in the rat

Level of dietary protein	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum	
			Total cholesterol, mg%	Lipid phosphorus, mg%
10% casein	6.1	29 ± 2*	1194 ± 136*	10.4
40% casein	9.0	108 ± 3	633 ± 71	6.6
10% fibrin	7.1	44 ± 5	988 ± 249	9.5
40% fibrin	9.7	107 ± 2	635 ± 63	7.7
10% pork†	7.0	32 ± 1	1271 ± 148	10.7
40% pork†	10.1	111 ± 3	733 ± 102	8.4
10% Drackett‡	6.3	12 ± 2	1742 ± 334	14.6
40% Drackett‡	10.6	117 ± 5	403 ± 55	5.3
40% wheat gluten	8.0	58 ± 3	210 ± 17	4.3
40% zein§	7.2	35 ± 3	1420 ± 228	11.3
10% casein + 30% zein	6.7	47 ± 6	821 ± 84	7.0
20% casein + 20% zein	9.2	108 ± 3	622 ± 52	7.2

*Standard error of the mean (S. E. M.).
†Extracted with petroleum ether.
‡Purified soya bean protein.
§Supplemented with 0.5% of DL-tryptophan and 2.5% of L-lysine-monohydrochloride.

ment. Such variations from experiment to experiment in the serum cholesterol concentration for a particular group were frequently observed; however, the relative values in duplicate experiments were always similar. Also, for each protein studied, the serum cholesterol concentration was lower when the diet contained 40% than when it contained 10% of protein. There were no significant differences among the serum cholesterol concentrations of rats fed casein, fat-free pork, or fibrin at the 10% level. This was also true of the values for comparable groups fed these proteins at the 40% level. The serum cholesterol concentration of the group fed the purified soybean protein[2] at the 10% level was higher than that of any other group, but when this protein was fed at the 40% level, the serum cholesterol concentration fell below those of the groups fed on diets that contained animal proteins.

The serum cholesterol concentration of the group fed zein, another vegetable protein, at the 40% level (supplemented with 0.5% of DL-tryptophan and 2.5% of L-lysine-monohydrochloride) was elevated above those of the other groups receiving a comparable level of protein. This hypercholesteremic effect of zein was counteracted when casein was substituted for part of the zein. The ingestion of a diet containing 20% of zein and 20% of casein led to a serum cholesterol concentration (622 ± 52 mg%) that was not significantly different from the value for rats fed a diet containing 40% of casein (633 ± 71 mg%). The ingestion of a diet containing 40% of wheat gluten as the dietary protein, like the ingestion of the diet containing zein, resulted in reduced food intake and retarded growth. However, unlike the zein regimen, it caused a marked lowering of serum cholesterol concentration, the value of 210 ± 17 mg% being lower than that for any other group.

None of these dietary changes, however, had any appreciable effect on the concentrations of total lipids, total cholesterol, and lipid phosphorus in the liver.

In contrast to the curve obtained with increasing levels of casein (Fig. 1), the serum cholesterol concentration decreased with each increase in the dietary level of wheat gluten up to 68.5% (Table II) and the value in each case was

TABLE II

Effects of different levels of wheat gluten in the diet on serum cholesterol concentration in the rat

Level of wheat gluten, %	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum		Liver		
			Total cholesterol, mg%	Lipid phosphorus, mg%	Total lipids, %	Total cholesterol, %	Lipid phosphorus, mg%
10	3.4	-2 ± 2*	486 ± 38*	6.7	18.3	7.16	45
20	4.9	15 ± 1	274 ± 36	5.1	15.8	5.04	37
30	6.7	38 ± 2	250 ± 31	4.7	21.0	6.16	46
40	7.6	56 ± 6	210 ± 8	4.7	16.6	6.02	44
68.5	8.8	101 ± 5	167 ± 10	3.9	9.0	2.61	46
10†	3.5	-2 ± 1	554 ± 77	8.0	14.9	5.22	42
20†	7.9	50 ± 4	295 ± 4	4.7	18.0	6.76	37
30†	10.6	102 ± 4	260 ± 11	4.9	17.7	5.50	45

*S. E. M.
†Wheat gluten supplemented with 1% of L-lysine-monohydrochloride.

lower than that obtained with a comparable level of casein in the diet. With only 10% of wheat gluten in the diet, the serum cholesterol concentration was as low as that obtained with 40% of casein in the diet. With higher levels of wheat gluten in the diet, the serum cholesterol concentration approached a range usually seen only when cholic acid is omitted from the basal diet. Supplementing the diet containing wheat gluten with 1% of L-lysine-monohydrochloride enhanced food intake and stimulated growth but did not alter the serum cholesterol values (Table II).

Total cholesterol and total lipid concentrations in the liver were not lowered when wheat gluten was substituted for other proteins up to the 40% level in the diet. However, increasing wheat gluten to 68.5% of the diet brought about a marked lowering of both the total lipid and the total cholesterol concentrations of the liver.

The effects of different levels of wheat gluten on the serum cholesterol concentration of rats fed on diets from which cholic acid was excluded are

shown in Table III. For comparison, groups receiving casein as the dietary protein were also included. When the diet contained 1% of cholesterol, but no

TABLE III

Effects of different dietary levels of casein and wheat gluten on serum cholesterol concentration in rats fed on diets containing 1% of cholesterol, but no cholic acid

Level of protein	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum	
			Total cholesterol, mg%	Lipid phosphorus, mg%
10% casein	6.8	34±2*	326±21*	5.1
30% casein	9.1	101±3	204±21	4.2
69% casein	8.4	103±3	302±13	5.2
10% wheat gluten†	6.4	15±2	135±2	4.3
30% wheat gluten †	11.1	93±6	116±7	4.7
69% wheat gluten†	8.9	112±4	130±4	4.6

*S. E. M.
†Supplemented with 1% of L-lysine-monohydrochloride.

cholic acid, an increase in the dietary level of casein from 10% to 30% caused a lowering of the serum cholesterol concentration. Increasing the casein content of the diet to 69%, however, caused an elevation in the serum cholesterol concentration. When wheat gluten was substituted for 10% of casein in the diet, there was a marked reduction in the serum cholesterol concentration. Increasing the dietary level of wheat gluten from 10% to 30% further reduced the serum cholesterol concentration but when wheat gluten was fed at the 69% level, the serum cholesterol concentration was slightly elevated. However, the differences among the serum cholesterol concentrations of the groups fed wheat gluten at these three levels were small.

TABLE IV

Effects of different dietary levels of casein and wheat gluten on serum cholesterol concentration in rats fed on diets containing no cholesterol or cholic acid

Level of protein	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum	
			Total cholesterol, mg%	Lipid phosphorus, mg%
10% casein	6.6	37±2*	114±3*	4.5
30% casein	10.5	112±4	71±3	3.2
70% casein	8.8	100±3	91±7	3.4
10% wheat gluten†	6.6	14±2	105±6	4.3
30% wheat gluten†	10.8	89±2	120±7	4.5
70% wheat gluten †	10.2	126±6	99±3	3.8

*S. E. M.
†Supplemented with 1% of L-lysine-monohydrochloride.

The serum cholesterol concentration of rats fed on diets from which both cholesterol and cholic acid were omitted was minimal when the diet contained 30% of casein (Table IV). Higher or lower levels of dietary casein caused an elevation of the serum cholesterol concentration. On the other hand, when wheat gluten was substituted for casein at the 30% level in this type of diet an elevation in serum cholesterol concentration resulted. Increasing the level of wheat gluten in the diet to 70% or decreasing it to 10% depressed the serum

TABLE V

Effects of different combinations of casein and wheat gluten on serum cholesterol concentration in the rat

Level of dietary protein, %		Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum		Liver	
Casein	Wheat* gluten			Total cholesterol, mg%	Lipid phosphorus, mg%	Total lipids, %	Total cholesterol, %
25	0	10.1	112±3†	677±70†	8.1	16.0	6.09
20	5	10.3	121±3	521±31	7.5	16.8	6.50
15	10	10.4	123±2	489±51	7.3	15.9	6.28
10	15	10.6	121±2	392±57	6.0	16.0	6.17
5	20	10.6	114±5	335±33	5.5	17.7	5.97
0	25	8.6	67±4	211±12	5.0	23.8	6.64

*Supplemented with 1% of L-lysine-monohydrochloride.
†S. E. M.

cholesterol concentration but the values were still significantly higher than that for the group fed casein at the 30% level.

The results presented in Table V show that the hypercholesteremia that occurred when the diet contained casein and cholesterol and cholic acid could be diminished by replacing the casein in part with wheat gluten. The diminution in the serum cholesterol concentration was proportional to the amount of wheat gluten in the diet.

A report that wheat gluten increases the excretion of fecal fat by the rat (15) suggested that wheat gluten might interfere with cholesterol absorption. However, no differences were observed, under any of these dietary conditions, in total quantity of lipid in the feces or in the intensity of the color developed when the sterol concentration of this lipid was estimated by the method of Sperry and Webb (16). On the assumption that the diets did not alter the proportion of the various sterols excreted, this would suggest that total sterol excretion on these diets was essentially unchanged. Furthermore, there were no significant differences among these groups in the concentrations of total lipids, total cholesterol, and lipid phosphorus in the liver. Liver lipid phosphorus concentration in these groups varied from 38 to 44 mg% and these values lie within the normal range. It is noteworthy that feeding of cholesterol and cholic acid causes a considerable rise in the concentrations of total lipid and total cholesterol in the liver but does not influence the liver lipid phosphorus concentration.

TABLE VI

Comparative effects of supplemental amounts of wheat gluten, glutenin, or gliadin on serum cholesterol concentration in the rat

Dietary protein	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum	
			Total cholesterol, mg%	Lipid phosphorus, mg%
25% casein	8.8	95±3*	518±64*	6.0
25% casein + 10% wheat gluten	9.8	114±5	312±30	4.9
25% casein + 10% glutenin	9.8	110±4	591±64	6.4
25% casein + 10% gliadin	9.7	109±3	521±37	6.5
25% casein + 5% glutenin + 5% gliadin	9.3	111±3	655±43	6.6

*S. E. M.

Table VI shows that supplementation of the diet containing 25% of casein and cholesterol and cholic acid with 10% of wheat gluten led to a significant reduction in the serum cholesterol concentration. However, when supplementary amounts of either glutenin or gliadin or a mixture of the two were added to the diet, no reduction in the serum cholesterol concentration occurred and in some animals a slight elevation of serum cholesterol concentration was observed.

The failure of glutenin and gliadin to bring about a reduction in the serum cholesterol concentration, combined with the fact that an increase in the level of dietary wheat gluten up to 68.5% led to a progressive lowering of serum cholesterol concentration in the rat ingesting a hypercholesteremic diet, suggested that some factor(s) carried along with wheat gluten might be responsible for its cholesterol-lowering effect. Wheat gluten washed under running water, then with acetone still possessed cholesterol-lowering activity.

Extraction of wheat gluten with absolute ethanol in a soxhlet apparatus for several days yielded some lipid-like material which was effective in lowering serum cholesterol concentration. The results in Table VII show that the

TABLE VII

Effect of extraction of wheat gluten with absolute ethanol on serum cholesterol concentration in the rat

Dietary alterations	Av. daily food intake, g	Av. wt. gain (3 weeks), g	Serum	
			Total cholesterol, mg%	Lipid phosphorus, mg%
25% casein	9.0	98±3	630±49*	6.6
25% wheat gluten†	8.9	69±3	300±30	4.8
25% ethanol extracted wheat gluten†	7.4	54±2	915±80	7.2
25% casein + 5% ethanol extract from wheat gluten	9.4	119±5	321±37	5.5

*S. E. M.
†Supplemented with 1.3% of L-lysine-monohydrochloride.

substitution of wheat gluten extracted with ethanol, for the casein in the diet, caused an increase in the serum cholesterol concentration above that observed when a comparable level of casein was fed. The ethanol extract of wheat gluten fed at a level of 5% in the diet diminished the hypercholesteremia associated with the ingestion of casein. The serum cholesterol concentration observed when the ethanol extract was fed was comparable to that observed after the feeding of the diet containing 25% of unextracted wheat gluten. The ingestion of this extract led also to a small reduction in the concentration of cholesterol (from 6.5 to 4.7%) in the liver.

General Discussion

Both the amount and the nature of the protein in the diet have a marked effect on serum cholesterol concentration in the rat. The reduction in serum cholesterol concentration observed when the dietary level of some proteins was increased from 10% to 40% is in agreement with published reports that high

levels of protein in the diet lower blood cholesterol concentration (8, 9, 17, 18, 19, 20). The observation of Olson *et al.* (21) that a high protein intake is associated with higher serum cholesterol concentration in man may be a result of the difference in the species studied or in the type of diets employed. They used protein from animal sources in their high-protein diets and vegetable proteins from cereals, rice, and legumes in their low-protein diets. The lower serum cholesterol concentrations they observed in subjects fed on low-protein diets may have been caused by some cholesterol-lowering factor in the vegetable proteins or some hypercholesteremic factor in animal proteins. Also the effect of protein in their experiments was probably influenced by the low choline content of the diets.

The failure of Loewe *et al.* (11) and Keys and Anderson (10) to observe an effect of dietary protein on serum cholesterol concentration in rabbits and humans may be related to the quantities of protein in their diets. Even the high level of protein used by Loewe *et al.* was inadequate to meet the requirement of the rabbit, while in the studies of Keys and Anderson, the entire range of dietary protein was high (64–138 g per day).

The hypercholesteremic effects observed in animals fed the lower dietary levels of protein may be a result of deficiencies of certain amino acids. The results obtained with zein suggest this. Zein is a poorly balanced protein and when it was fed at a level of 40% in the diet, food intake decreased, growth was retarded, and there was a marked hypercholesteremia. The hypercholesteremic effect of zein was counteracted when it was replaced in part by casein. Methionine deficiency has been reported to cause hypercholesteremia (5, 6, 22) and such an effect might explain the greater hypercholesteremia observed when casein was replaced with Drackett protein at the 10% level in the diet.

The elevation of serum cholesterol concentration observed when the level of casein in the diet was raised from 40% to about 70% is in agreement with the observation of Fillios *et al.* (23), who noted a decrease in serum cholesterol concentration when the dietary level of protein was raised from 5% to 20% and a subsequent increase in cholesterol concentration when the protein level was further raised to 40 or 60%. Jones and Huffman (24) reported an optimum range of 12–18% of dietary protein for rats fed on diets from which cholic acid had been omitted, and Fillios *et al.* (23) reported that the most favorable level of protein varies with the source of carbohydrate. The response to protein probably depends upon the type of diet fed. The observation (25) that neither the level nor the nature of the dietary fat had any effect on the serum cholesterol concentration of chicks fed a high protein diet also suggests that the response to other factors may be influenced by the level and type of dietary protein.

The results of the experiments with wheat gluten suggest that the protective action of some vegetable proteins against atherosclerosis or hypercholesteremia (3, 4) may be attributable to the presence of some active component associated with these proteins. Preliminary experiments with some other supposedly purified vegetable proteins show that they also contain appreciable amounts of lipid-like material. The cholesterol lowering observed upon the feeding of Drackett protein in place of casein at the 40% level might be due to the lipid

material associated with this protein. However, experiments with Drackett protein and zein also suggest that either the quantity of the cholesterol-lowering component in these two proteins is low or else it does not possess great activity. The quantitative aspects of these effects of different vegetable proteins are under investigation.

These observations also emphasize that in nutritional experiments in which vegetable proteins are used, appreciable quantities of lipid material may be ingested along with the proteins, and, if the effects of various levels of such vegetable proteins are being studied, the calorie content of the diet will be increased when the protein level is raised. It therefore becomes important in such experiments to differentiate clearly between the effects of protein level, per se, and of a change in the calorie to protein ratio (26).

How this lipid material exerts its cholesterol-lowering effect is not clear. The inclusion of unextracted wheat gluten in a diet containing neither cholesterol nor cholic acid actually caused serum cholesterol concentration to rise, therefore, it seems unlikely that this material would prevent hypercholesteremia of endogenous origin. Nor does it appear to interfere with the absorption of cholesterol in the gut because the feeding of wheat gluten did not increase the fecal excretion of either lipid or Liebermann-Burchard-positive chromogens. The lipid material may contain plant sterols but the reduction in the serum cholesterol caused by feeding β-sitosterol is accompanied by a simultaneous reduction in liver cholesterol (27), whereas the feeding of 40% of unextracted wheat gluten in the diet considerably reduced the serum cholesterol concentration without affecting the deposition of cholesterol in the liver. It has been reported by Beveridge *et al.* (28) that the cholesterol-lowering effect of corn oil may depend upon the presence of several different components. It is likely that the lipid material associated with wheat gluten is also a complex mixture. The nature and properties of this lipid material are being studied and attempts are being made to separate fractions and to study their modes of action. The factor appears to affect hypercholesteremia of exogenous origin only.

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NOTES

LACK OF EFFECT OF INCREASING ILLUMINATION ON THYMUS WEIGHT IN RATS AND PIGEONS

E. O. HÖHN

In an earlier publication (1) a post-breeding-season enlargement of the thymus in several species of wild birds was demonstrated and a similar summer enlargement of the thymus in mule deer has also been described (2). The observers of the phenomenon in mule deer noted a correlation between day length and thymus volume. Similarly, some unpublished observations by Farner (3) showed a progressive increase of thymus weight in castrated white-crowned sparrows exposed to increasing illumination. These observations suggested that light, possibly acting via the pituitary, might have a thymotropic action and that such an effect might explain seasonal thymus cycles in birds and mammals.

To test this hypothesis, ten 3-month-old virgin female Sprague–Dawley rats and 10 ovariectomized rats of the same age and strain were subjected to increasing illumination and compared with an equal number of normal and ovariectomized controls. Control animals were kept at a constant illumination of 8 hours per day. The experimental animals at the end of the 8-hour daily illumination period, which they shared with the controls, received light from a 40-watt fluorescent light suspended 3 feet above the animal cages, for half an hour. The period of added illumination of the experimental animals was increased daily by half an hour until they were under continuous lighting and this was maintained for an additional 45 days. Experimental and control animals were then killed and weighed and the thymi were dissected and weighed. Mean thymus weights, expressed as per cent of body weight in the various groups, were as follows:

Control ovariectomized (10 animals)	0.171
"Illuminated" ovariectomized (10 animals)	0.172
Control virgin females (10 animals)	0.107
"Illuminated" virgin females (10 animals)	0.096

It is obvious that illumination had no significant effect on thymus weight in either the ovariectomized or the normal female rats.

A similar experiment was performed on castrated 6-month-old domestic pigeons. Three control and three experimental birds were used. Mean thymus weight expressed as per cent of body weight was 0.110 in the control and 0.115 in the "illuminated birds". Although the sample is too small to be conclusive, the results certainly fail to suggest any effect of illumination on thymus weight. In view of these findings in pigeons, Farner's observation of a weight increase in castrated "illuminated" birds may have been due to the fact that he used wild birds as controls. These may have been subjected to stress factors causing some degree of thymic atrophy from which the captive birds were protected.

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INSTRUMENT FOR WITHDRAWAL AND PROPORTIONING OF FLUIDS*

JAMES CAMPBELL

With the instrument described, blood can be withdrawn and several aliquots delivered for analysis. The transfer of the blood to a container and pipetting of samples are thereby eliminated. One of these instruments consists of a 1-ml syringe and the attachments shown in the figure. A knife-blade (A) is

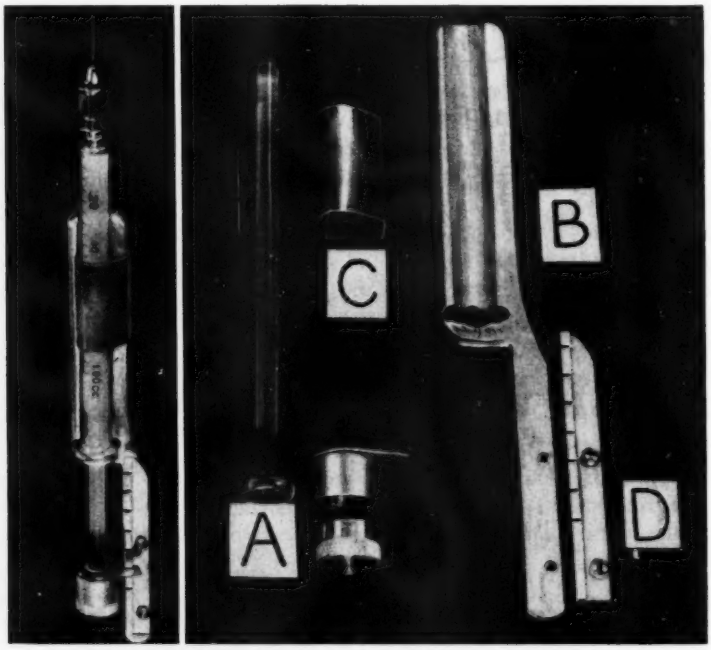

FIG. 1. The assembled instrument is shown on the left and the parts on the right.

fixed to the knob of the plunger at right angles to the long axis and the barrel of the syringe is secured by a bronze clip (C) to the holder (B) made from a

*Used in investigations aided by a Grant from the National Research Council of Canada.

single piece of aluminum. To prevent movement, the collar of the syringe must fit snugly into the aperture in B. The knife-blade on the plunger fits into the accurately positioned notches machined on a separate L-strip of metal (D) whose connection to the holder is adjustable. The complete assembly weighs 44 g. To give delivery of the required volume of fluid when the plunger is moved from one notch to the next, the distance between the notches can be calculated from the inside diameter of the syringe. A calibration of an instrument for delivery of 0.10-ml portions is shown in the table. The volumes delivered by moving the plunger over any two adjacent notches are in good

TABLE I
Calibration

Notch Nos.	No. of observations	Mean volume delivered, μl $\pm$ std. dev.
7–6	6	100.3 ± 0.27
6–5	7	99.5 ± 0.53
5–4	6	99.8 ± 0.42
4–3	7	99.7 ± 0.17
3–2	8	100.4 ± 0.41
2–1	7	99.7 ± 0.25
1–0	6	99.0 ± 0.25

NOTE: A 1-ml syringe mounted as shown in the photograph was calibrated with mercury.

agreement; the errors so far have been in the positioning of the notches, i.e. the variations were due to the construction rather than to manipulation of the instrument.

In the determination of blood sugar (1) for example, blood is withdrawn into the assembled instrument, the syringe and needle of which have been moistened with a mere film of heparin solution, 1000 International Units per milliliter. If desired, an anticoagulant can be eliminated since the time required for delivery is so brief. A piece of gauze is now placed over the upright needle, bubbles are expressed, the knife-blade of the plunger is moved into a notch, and the needle is wiped with the gauze. Then 0.10-ml aliquots of blood may be introduced directly into 3.5 ml of water in 15×80-mm test tubes.

The same design can be adapted for lesser or larger volumes of fluid. An instrument to hold a 0.25-ml syringe and to deliver 0.05 ml of blood or other fluid has been used.

The instruments were made by Mr. E. B. Johnson, Senior Technician of this Department.

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THE EFFECT OF SULPHONYLUREA DRUGS ON PANTOTHENIC ACID METABOLISM IN TETRAHYMENA PYRIFORMIS

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While screening drugs which are used over extended periods in man, for possible drug–vitamin interrelationships, it was found that tolbutamide and two other oral antidiabetic drugs markedly inhibited growth of *Tetrahymena pyriformis* in media containing suboptimal concentrations of pantothenic acid. Propylthiouracil was the only other drug among over 50 tested which appeared to interfere with pantothenic acid metabolism in this protozoan.

The basal medium was that of Dewey *et al.* (1) except for the concentration of the vitamin under test. Sterile solutions of vitamins and drugs were added aseptically to the medium, and the total volume was made up to 4.0 ml in each tube. The inoculum was prepared from a 3-day-old washed culture of *Tetrahymena pyriformis* W. The washed cells were diluted (1 to 10) in single strength basal medium and 0.2 ml of this suspension was used as the inoculum. Cultures were incubated in 20-mm tubes held in a sloped position at 25° C for 72 hours. Growth was measured turbidimetrically with the Coleman Spectrophotometer Model 11. In preliminary tests inhibition curves were established for each drug in the complete medium and subsequently drugs were tested at concentrations which were then known to be non-inhibitory in the complete medium. The concentrations of vitamins in the complete medium were considered optimal (concentrations in the medium of Dewey *et al.* (1)); the optimal concentration of calcium pantothenate was 1.0 μg/ml.

Some of the data from the initial screening tests for drug–vitamin interrelationships are shown in Table I. The sulphonylurea drugs, tolbutamide,

TABLE I

Effect of certain antidiabetic and antithyroid drugs on the growth of *Tetrahymena pyriformis* with suboptimal amounts of vitamins

Drug	Level, μg/ml	Niacin		Pyridoxal		Pantothenic acid		Thiamine	
		S.*	O.†	S.	O.	S.	O.	S.	O.
		Optical density × 100‡							
Experiment 1									
None	—	29	64	26	72	18	70	42	62
Tolbutamide	50	24	66	22	62	2	66	42	62
Metahexamide	100	28	62	21	61	2	64	37	51
Chlorpropamide	100	24	66	20	70	2	72	39	60
Phenformin	10	29	74	24	70	26	72	43	68
Experiment 2									
None	—	62	80	30	72	52	78	42	62
Propylthiouracil	25	62	72	19	70	22	72	40	57
Pantoyltaurine	1000	—	—	—	—	52	78	—	—

*Suboptimal amount.
†Optimal amount.
‡A measure of culture turbidity (2-log galvanometer reading).

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metahexamide, and chlorpropamide, had little or no effect on the growth of the protozoan in a medium containing optimal amounts of all B vitamins, but markedly inhibited growth when the medium contained suboptimal amounts of pantothenic acid. Phenformin, a non-sulphonylurea oral antidiabetic drug, had little or no effect on growth of the test organism. The effects of propylthiouracil and pantoyltaurine on growth of the protozoan are shown in experiment 2. The suboptimal levels of vitamins tested in this experiment were higher than those used in experiment 1. The inhibitory effect of propylthiouracil seemed to be reversed by pantothenic acid. Pantoyltaurine (a pantothenic acid antagonist for some bacteria) at a concentration of 1 mg per ml had no apparent antivitamin effects.

The pantothenic acid – drug interrelationships were tested over a wider range of concentrations of both drugs and vitamins; some of the data obtained with metahexamide are given in Table II. Concentrations of 100 μg or more

TABLE II

Reversal by pantothenic acid of the inhibitory effect of metahexamide on the growth of *Tetrahymena pyriformis*

Pantothenic acid, mμg/ml	Metahexamide, μg/ml				
	0	20	40	100	200
	Optical density × 100*				
0	0	0	0	0	0
10	16	2	1	0	0
15	28	3	1	1	0
30	47	12	6	3	1
50	58	33	14	4	2
100	60	53	42	12	4
1000	63	61	64	61	58

*See footnote Table I.

of metahexamide per milliliter almost completely inhibited growth with the minimum concentration of pantothenate (50 mμg per ml) normally required for essentially maximum growth of *T. pyriformis*. The inhibition with 200 μg per ml of the drug, however, was almost completely reversed when the medium contained 1 μg of pantothenic acid per milliliter. Tolbutamide, chlorpropamide, and propylthiouracil also produced similar growth inhibitions which were effectively reversed by similar concentrations of pantothenic acid.

It is of interest that both propylthiouracil, which inhibits the synthesis of thyroxine, and tolbutamide, which prevents the glycogenolytic effect of thyroxine (2), interfere with pantothenic acid metabolism in *T. pyriformis*. Further studies will be conducted to determine whether the sulphonylureas influence pantothenic acid metabolism in higher animals.

1. Dewey, V. C., Heinrich, M. R., and Kidder, G. W. J. Protozool. **4**, 211 (1957).
2. Kaldar, A. and Pogatsa, G. Lancet, **21**, 386 (1959).

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NOTE ON THE MEASUREMENT OF TRANSLOCATION OF PHOTOSYNTHATE IN VERY SHORT TIMES

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Nelson *et al.* (1) have reported in this Journal a very rapid translocation of photosynthate in soybean when a large dose of $^{14}CO_2$ is applied for a short time to one of the primary leaves. This fact, if true, is of the greatest interest in translocation studies but the validity of the experiment is open to question on the grounds of a leakage from the application chamber. They record that $^{14}CO_2$ was injected into "a small polyethylene bag which was sealed around one of the primary leaves of the plant". They reject the possibility of escaped gas being fixed in remote parts of the plant to give counts in their subsequent extracts on the grounds that a neighboring plant showed no fixed radioactivity. This seems good evidence but becomes more doubtful if we consider how much $^{14}CO_2$ must have escaped from the bag.

In our work, polyethylene has been discarded as a suitable material for application chambers for $^{14}CO_2$ because of the high permeability of polyethylene to this gas (8.64 ml gas/sq. m/24 hr/cm Hg/mm thickness (2)). Taking as a reasonable assumption that the bag used was made of film four-thousandths of an inch thick (a common gauge of moderate thickness), this unit becomes

$$\frac{8.64}{48\times60}\ \mu l/cm^2/30\ sec/cm\ Hg/.004\ in.\ thickness.$$

Thirty seconds was the time during which the application was made. Now let us assume a bag 8×10 cm, having a surface area of 160 cm^2, slightly distended to give a volume of 50 ml. The injected 50 μc of $^{14}CO_2$ had a specific activity of 8.86 mc/mmole and was equivalent to 50/8.86 μmole CO_2, which would exert at standard temperature in a 50-ml volume, a pressure of

$$\frac{76\times50\times22.4\times10^{-3}}{8.86\times50}\ cm\ Hg.$$

The correction to room temperature is not worth making.

So the gas passing through the walls in 30 seconds is

$$\frac{8.64\times160\times76\times22.4\times10^{-3}}{48\times60\times8.86}\ \mu l$$

$$=\frac{38\times10^{-3}}{9}\ \mu mole\ CO_2\ at\ S.T.P.$$

The specific activity of the injected gas was 8.86 μc/μmole, so the calculated leak has a radioactivity of

$$\frac{38\times8.86\times10^{-3}}{9}\ \mu c$$

which produces

$$\frac{38\times8.86\times10^{-3}\times3.7\times10^{4}\times60}{9} \text{ disintegrations per minute}$$

$$= 8.5\times10^{4} \text{ d.p.m.}$$

This quantity of radioactivity then will be lost through the walls of the bag in the course of the experimental run and can be carried by draughts to any green part of the plant and there fixed, since the plants were "under illumination of 2000 ft-c." This seems sufficient stray radioactivity to account for the small amounts they record in remote parts (all green), of the order of 10–30 d.p.m., a very small fraction of the calculated leak. Dispersal by draughts might also explain the curious gaps in their series of extracts where parts remote from the application show radioactivity and intermediate parts do not. It is possible also that the control plant nearby which gave no observable counts was not in the draught.

The obvious precaution against fixation of gas-borne $^{14}CO_2$, of darkening all the plant except the application leaf, would have to be checked in *Soya* lest the presence of a dark CO_2-fixation system should vitiate the results. The presence of ^{14}C in root extracts would be more conclusive.

This note is not intended to disparage this most interesting work but merely to point out the reasons which prevent acceptance of the evidence as presented.

My thanks are due to the Directors of Imperial Chemical Industries of Australia and New Zealand for permission to publish this note.

1. Nelson, C. D., Perkins, H. J., and Gorham, P. R. Can. J. Biochem. and Physiol. **36**, 1277 (1958).
2. "Alkathene" Brand of Polythene. Booklet issued by ICI Ltd. 1950.

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Canny criticizes the experiments demonstrating rapid translocation of photosynthetically assimilated C^{14} out of the primary leaf of the soybean on the grounds of leakage of $C^{14}O_2$ from the polyethylene-film application chamber. The second of the three controls mentioned in the original note was designed to test this point. It was found that "no radioactivity could be detected in the stem of an untreated plant which was growing in the same pot as the treated plants during the experiment." Although it was not stressed in the original note, the experiments were carried out in a strong up-draught in a fume hood. Therefore, it is unlikely that the $C^{14}O_2$ that leaked from the chamber could have been dispersed by draughts in such a way that parts of the stem remote from the application fixed $C^{14}O_2$ while intermediate parts did not. Work that is now in press (Nelson, C. D. *et al.* Can. J. Botany, **37**, 1181 (1959)) further refutes Canny's criticism. The original experiments have been repeated and confirmed using a lucite chamber (Towers, G. H. N. and

Mortimer, D. C. Can. J. Biochem. and Physiol. **34**, 511 (1956)) where leakage was impossible. Canny also suggests that the presence of C^{14} in root extracts would be more conclusive evidence for the existence of rapid translocation. After 30 seconds, C^{14} has indeed been isolated from the roots of plants grown in solution culture (Nelson, C. D. *et al.* Can. J. Botany, **37**, 1181 (1959)). Moreover, when the time of translocation was increased from 30 seconds to 10 minutes, there was an increase in the C^{14} content of the root without any increase in the C^{14} content of the hypocotyl.

It is concluded that rapid translocation of C^{14} is not an artifact due to leakage of $C^{14}O_2$ from the application chamber.

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